CHAPTER 3
RESULTS AND DISCUSSION

3.1 Primary Bioassay Screening Results of Crude Extracts.

3.1.1 Antioxidant test

Two methods to screen for antioxidants in crude plant extracts was used. Qualitative method of testing were by use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as a TLC spray reagent (TLC autographic assay). The other one was quantified in a spectrophotometric assay at λ_{max} 516 nm.

Table 3.1 Qualitative Antioxidant Results from TLC autographic assay of various crude extracts

Crude extracts	Antioxidant
Hexane	Negative
Dichloromethane	Positive
Ethyl acetate	Positive
Methanol	positive

Table 3.2 Quantitative Antioxidant Results from UV absorbance at λ 516 nm. of various crude extracts

Crude extracts	Conc. (ppm)	UV absorbance				%
		1	2	3	Average	
CH_2Cl_2	500	0.12639	0.13136	0.13020	0.12932	93.28
	250	0.23366	0.27322	0.24666	0.25118	86.95
	100	0.93669	0.95248	0.92920	0.93946	51.20
	50	1.47382	1.39056	1.37242	1.41226	26.64
EtOAc	500	0.16045	0.16747	0.16396	0.16396	91.48
	250	0.18941	0.19102	0.18166	0.18736	90.27
	100	1.07185	0.94902	0.94812	0.98966	48.59
	50	1.35837	1.40468	1.41171	1.39159	27.71

MeOH	500	0.43414	0.43810	0.46024	0.44416	76.93
	250	0.28215	0.27621	0.28294	0.28043	85.43
	100	0.82629	0.80119	0.80696	0.81148	57.85
	50	1.38992	1.36940	1.33255	1.36395	29.15
DPPH	0.3 mM	1.91212	1.93011	1.93292	1.92505	-

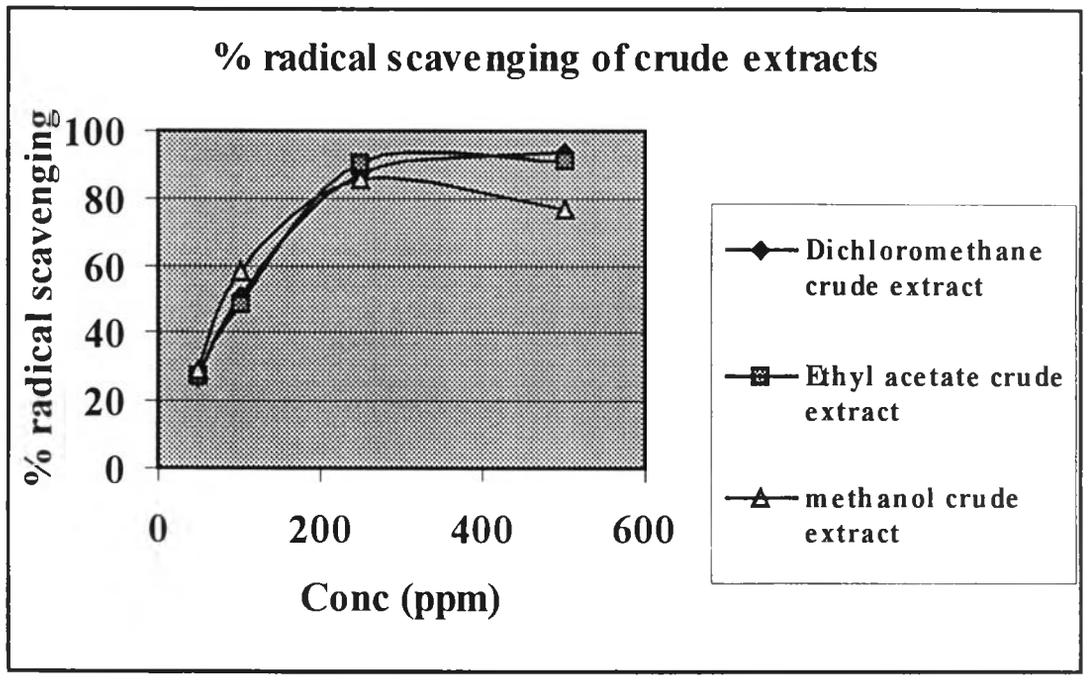


Figure 3.1 Scavenging effect of crude extracts

Table 3.3 The efficient concentration (IC_{50}) of crude extracts

Crude extracts	IC_{50} (ppm)
Hexane	NT*
Dichloromethane	118.95
Ethyl acetate	117.21
Methanol	87.03

NT* = not test

IC_{50} is the concentration that inhibited 50% of coloration

3.2 Properties and Structure Elucidation of Isolated Compounds

3.2.1 Compound 1

Compound 1 was isolated as white needles (0.2701g), from open column chromatography of dichloromethane crude extract. Its melting point was 212-213 °C (lit[22] 215-216 °C) and Rf value was 0.50 (SiO_2, EtOAc-CH_2Cl_2, 1:9). It was soluble in ethyl acetate and methanol but not in Hexane and CH_2Cl_2 .

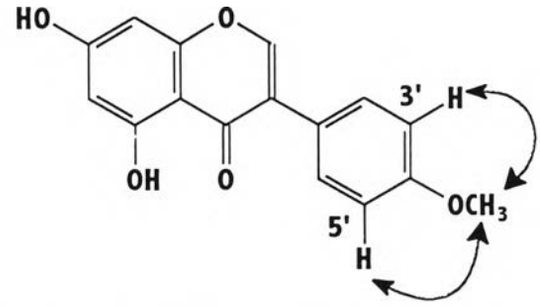
The IR spectrum (**Figure 3.2**) indicated hydroxy groups at ν_{max} 3200-3600 cm^{-1}(broad), the strong absorption band at ν_{max} 1659 cm^{-1} revealed the presence of α,β-unsaturated carbonyl and the characteristic absorption peak due to an aromatic moiety was observed at ν_{max} 1621, 1573, 1519, and 1438 cm^{-1}.

The Mass spectrum (**Figure 3.3**) of Compound 1 showed a molecular ion peak at m/z 284 [M^+], and its molecular formula was determined as $C_{16}H_{12}O_5$ by EIMS, ^1H and ^{13}C NMR data.

The ^1H NMR spectrum (**Figure 3.4**) displayed signals for para disubstituted aromatic protons at δ 7.47 (2H, *d, J = 8.85 Hz*) and 6.97 (2H, *d, J = 8.85 Hz*) ppm, two meta aromatic protons at δ 6.36 (1H, *d, J = 2.14 Hz*) and 6.21 (1H, *d, J = 2.14 Hz*) ppm, corresponding to each 2H protons. This observation implied the characteristics of unsubstituted benzene ring and three singlet signals at 12.90 (proton signal disclosed low field, this hydroxy formed hydrogen bond [δ 12.90] with carbonyl group but another one no hydrogen bond, displayed upper field than [δ 8.31]), 8.31 (broad) and the presence of methoxy group was indicated by singlet signal at δ 3.76 ppm.

The ^{13}C NMR spectra (**Figure 3.5**) showed sixteen carbon signals (flavonoid characteristic). The spectrums of DEPT 90 and 135 of this compound shown in **Figure 3.6**, Compound 1 had one carbonyl group at δ 180.1 ppm, one methoxy carbon at δ 55.1 ppm, seven methine carbons at δ 154.2 (C-2), 130.1 (C-2',6'), 113.7 (C-3',5'), 99.0 (C-6), and 93.7 (C-8), and seven quarternary carbons at δ 164.4 (C-7), 162.0 (C-5), 159.1 (C-4'), 157.6 (C-9), 122.9 (C-1'), 121.9 (C-3), and 104.4 (C-10).

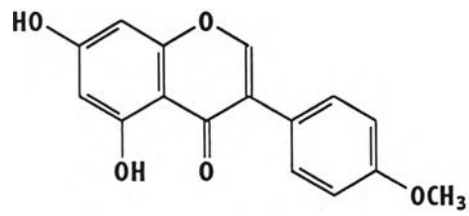
The position of a substitution (methoxy) group was also confirmed by the NOE difference technique. When the methoxy group (δ 3.77 ppm) was irradiated (**Figures 3.7 and 3.8**), NOE was observed in two protons at δ 6.98 (H-3',5') which showed a NOE on irradiation of the protons at C-3',5'.



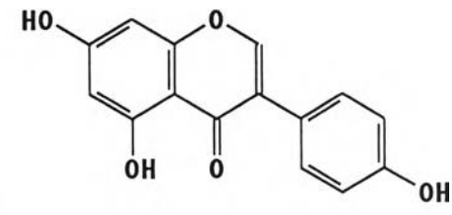
The NOE DIFF correlation of compound 1

Table 3.4 ^{13}C NMR data of compound 1 compared with genistein.

Positions	Compound 1	Genistein[23]
2	154.2	153.6
3	121.9	121.4
4	180.1	180.2
5	162.0	157.6
6	99.0	98.6
7	164.4	164.3
8	93.7	93.7
9	157.6	157.6
10	104.4	104.6
1'	122.9	122.4
2'	130.1	130.0
3'	113.7	115.2
4'	159.1	162.1
5'	113.7	115.2
6'	130.1	130.0
$-OCH_3$	55.1	-

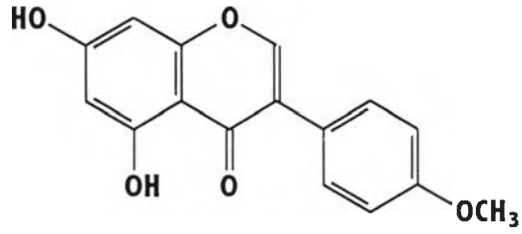


Biochanin A

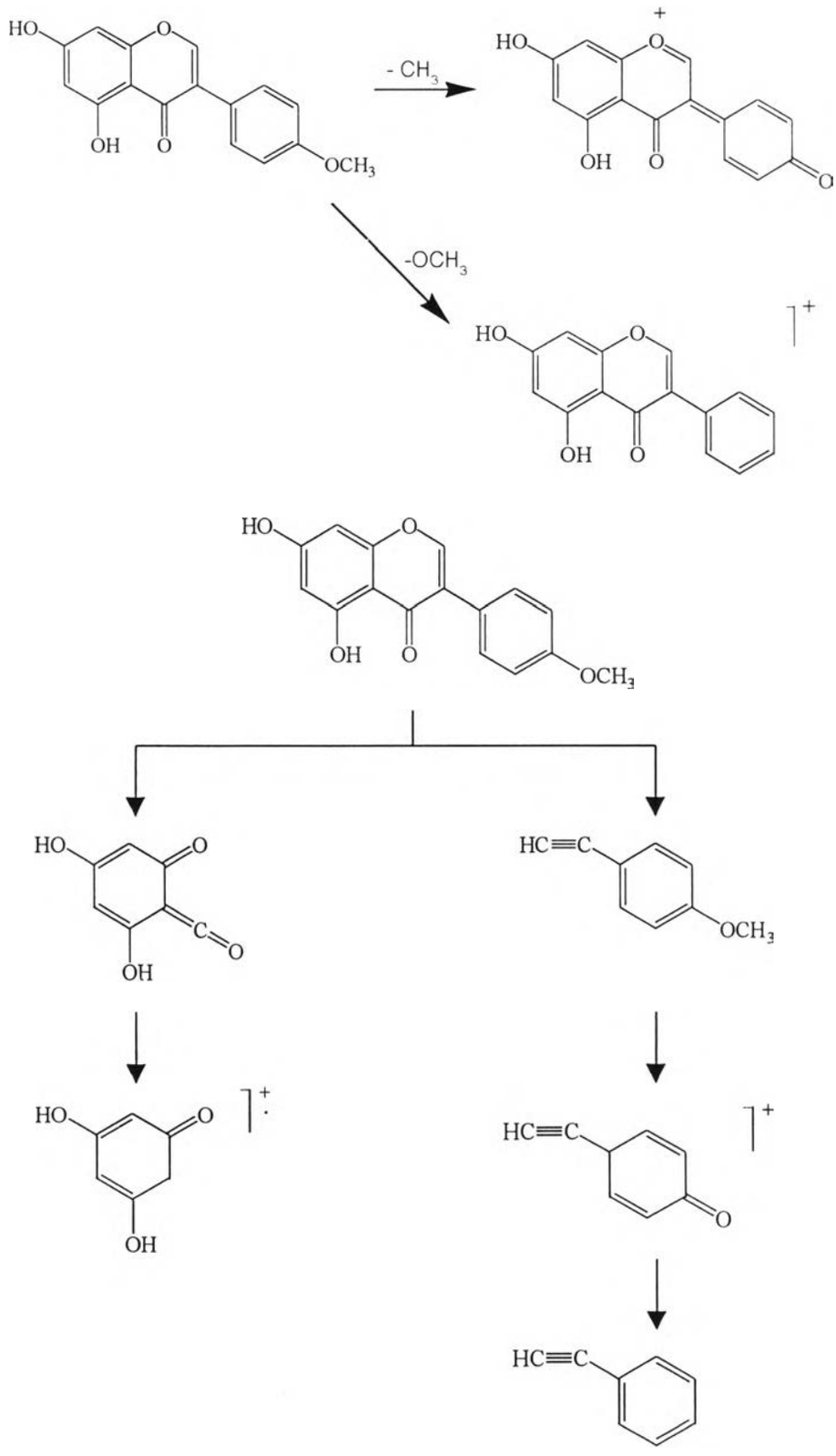


Genistein

From the spectral data, this compound was established as 5,7-dihydroxy-4'-methoxyisoflavone or Biochanin A.



The structure of compound 1



Scheme 3.1 Possible mass fragmentation patterns of compound 1

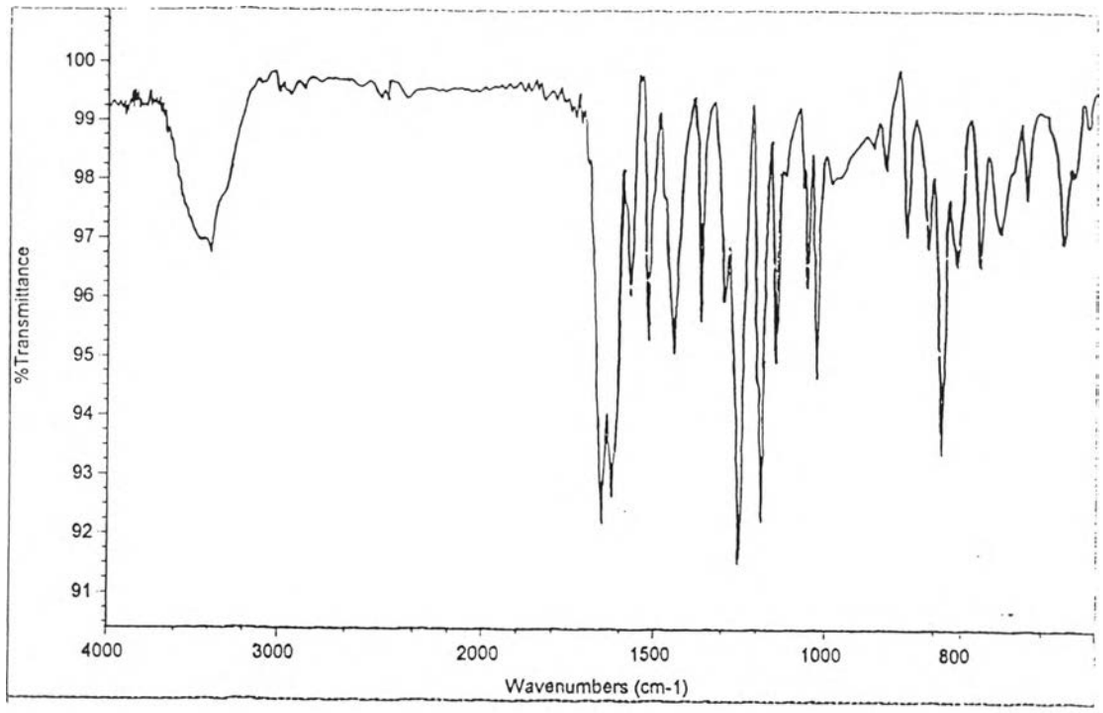


Figure 3.2 IR spectrum of compound 1

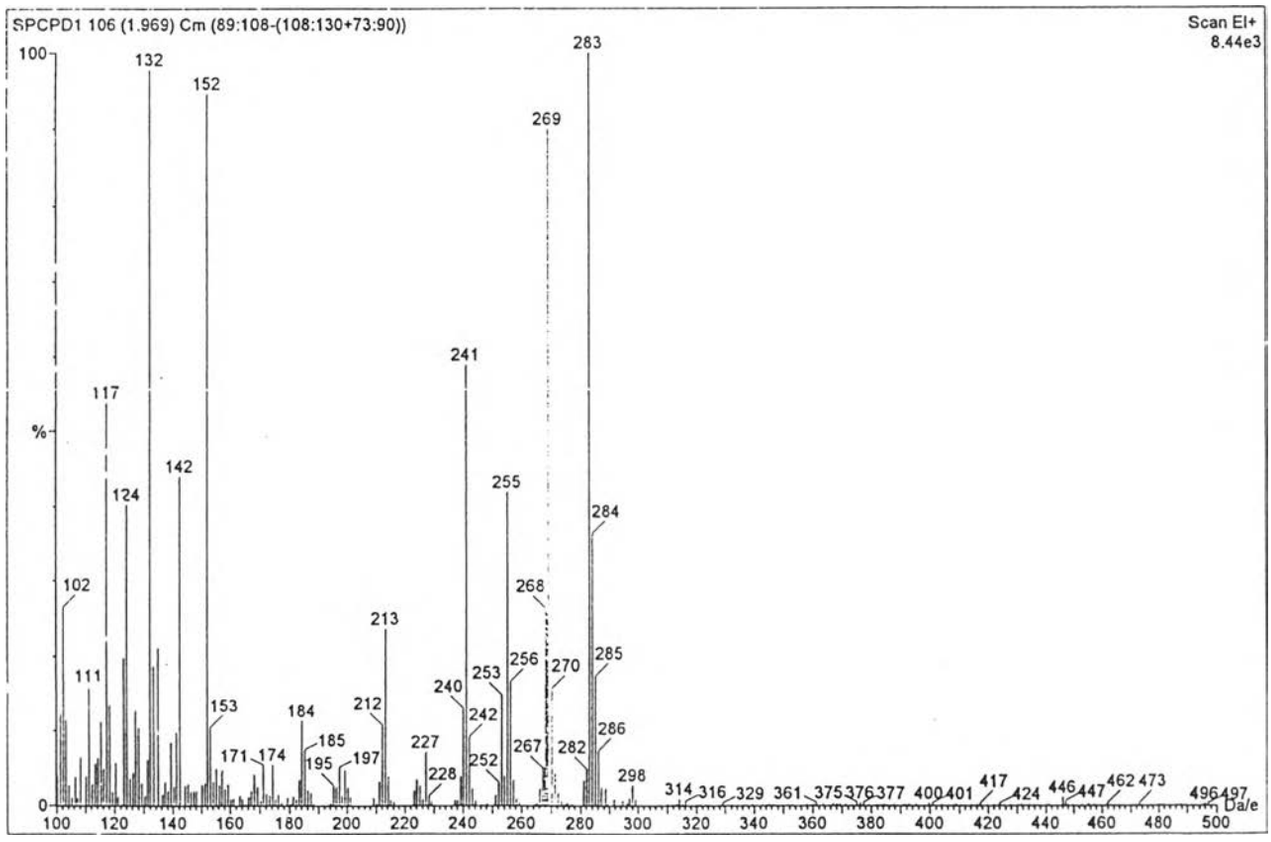


Figure 3.3 Mass spectrum of compound 1

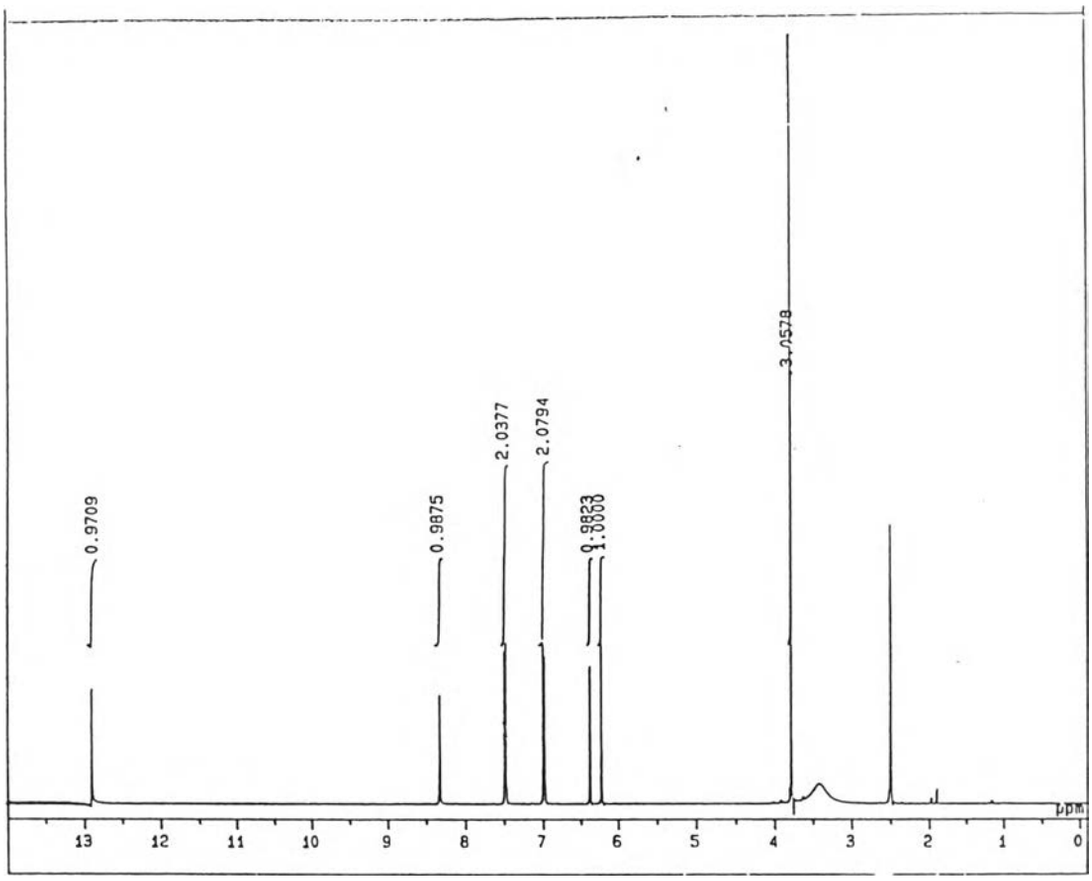


Figure 3.4 ^{1}H-NMR spectrum of compound 1

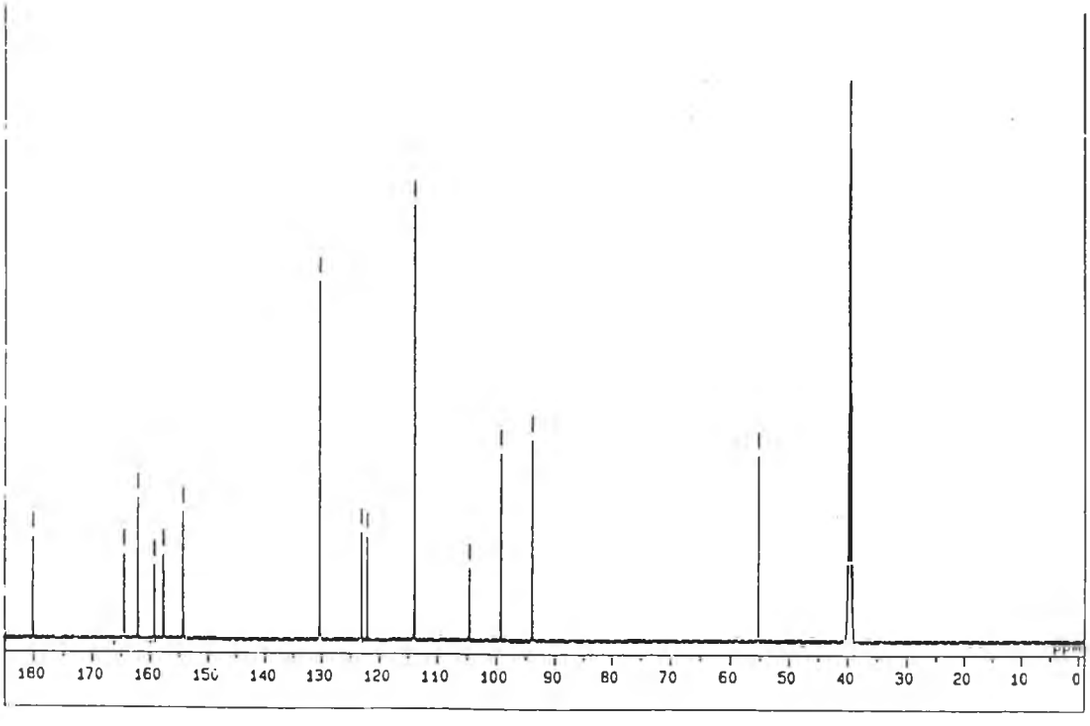


Figure 3.5 ^{13}C-NMR spectrum of compound 1

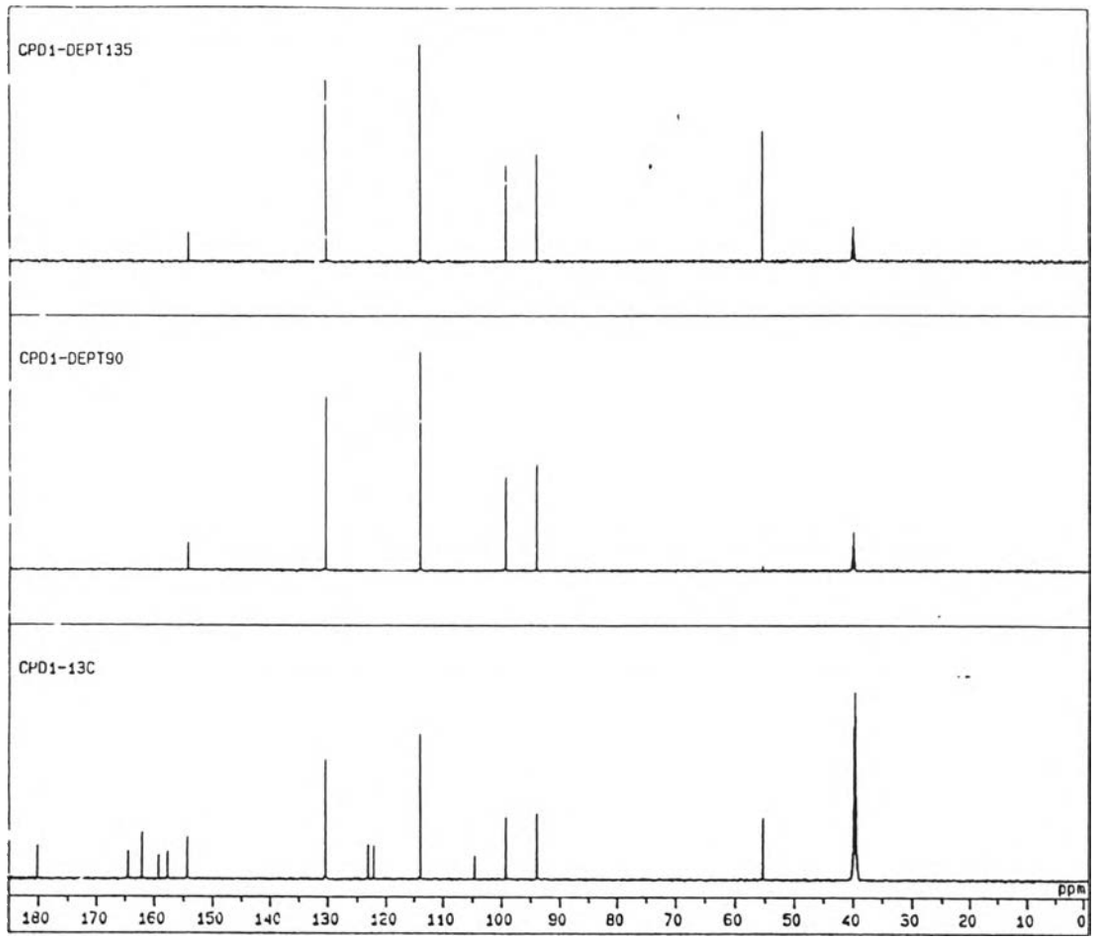


Figure 3.6 DEPT 90 and 135 spectrums of compound 1

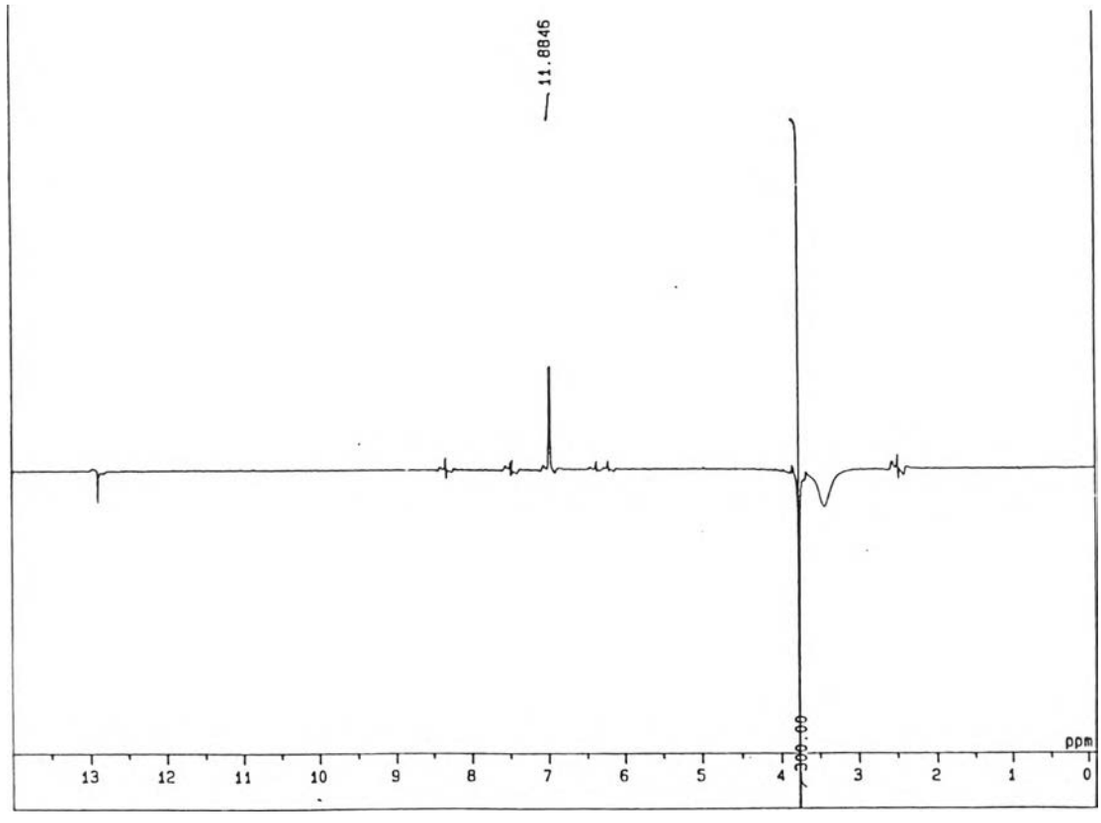


Figure 3.7 NOE DIFF of compound 1 (irradiated at 3.77 ppm)

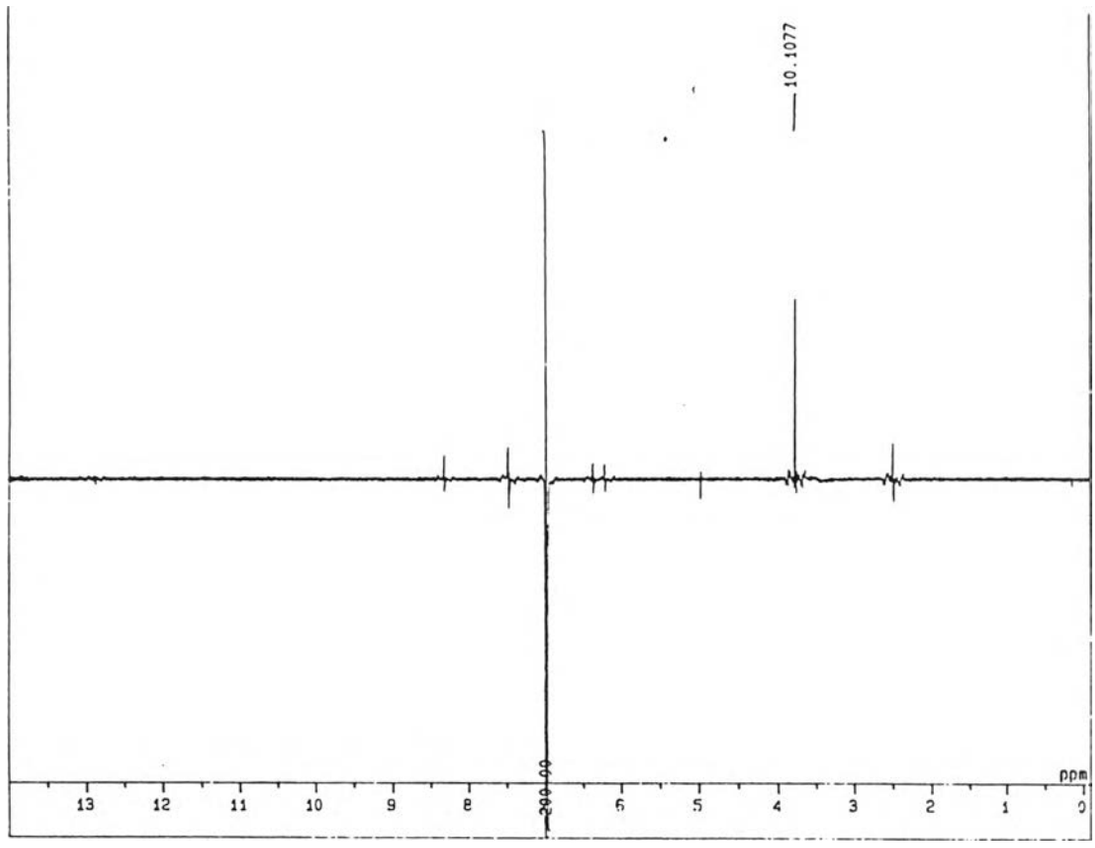


Figure 3.8 NOE DIFF of compound 1 (irradiated at 6.98 ppm)

3.2.2 Compound 2

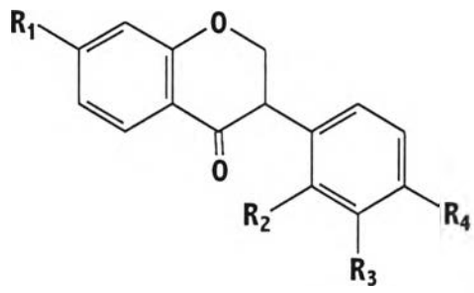
Compound 2 was obtained as white needles (0.3230g) from dichloromethane crude extract, after recolumn-chromatography and recrystalization in $MeOH/CH_2Cl_2$, with melting point 207-209 °C (lit[24] 200-202 °C) and Rf 0.19 (SiO_2, EtOAc-CH_2Cl_2, 1:9). It was soluble in methanol and slightly in ethyl acetate but not in hexane and dichloromethane.

The IR spectrum (**Figure 3.9**) clearly confirmed the presence of hydroxy group at ν_{max} 3200-3600 cm^{-1} due to O-H stretching vibration, carbonyl group at ν_{max} 1664 cm^{-1} due to C=O stretching vibration and benzene moiety at ν_{max} 1589, 1497, and 1450 cm^{-1}.

The mass spectrum (**Figure 3.10**) of compound 2 exhibited peaks at m/z 316 [M^+], 285 [M^+-CH_3O], 255 [M^+-$C_2H_5O_2$]. From EIMS, ^1H and ^{13}C NMR data, compound 2 was determined to be $C_{17}H_{16}O_6$.

The ^1H NMR (CD_3OD) spectrum (**Figure 3.11**) gave five signals of aromatic protons at δ 7.66 (1H, *d*, *J = 8.55 Hz*), 6.65 (1H, *d*, *J = 8.85 Hz*), 6.53 (1H, *d*, *J = 8.55 Hz*), 6.50 (1H, *dd*, *J = 8.55, 2.44 Hz*), 6.32 (1H, *d*, *J = 2.44 Hz*), one methine proton at δ 4.08 (1H, *dd*, *J = 5.49, 5.19 Hz*), one methylene proton at δ 4.50 (1H, *d*, *J = 11.29 Hz*), and 4.41 (1H, *dd*, *J = 5.50, 5.49 Hz*), two methoxy group at resonances 3.75 (3H, *s*), and 3.64 (3H, *s*).

The ^{13}C NMR spectra (**Figure 3.12**), DEPT 90 and 135 spectra (**Figure 3.13**) of this compound indicated seventeen carbons of fifteen skeleton and two substituent groups (characteristic of flavonoid); two methyl carbons of methoxy groups at δ 56.6 (OCH_3) and 60.4 (OCH_3) ppm, six methine carbons at δ 49.6, 103.6, 107.8, 111.7, 120.8, 130.3 ppm, one methylene carbon at δ 72.4 ppm, eight quarternary carbons at δ 115.6, 123.0, 140.7, 147.4, 150.0, 165.7, 166.4, and 194.4 ppm (C=O, characteristic of the isoflavanone). All of the above data suggested the possibility of a flavonoid skeleton of isoflavanone type. Therefore, the data and comparison with ^1H, ^{13}C NMR of known isoflavanone suggests that the molecule of this compound must have the basic skeleton shown as follows:

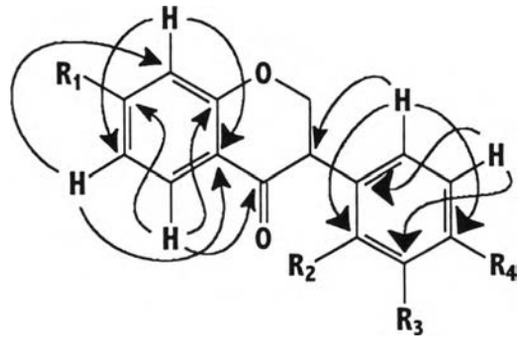


Substituents:

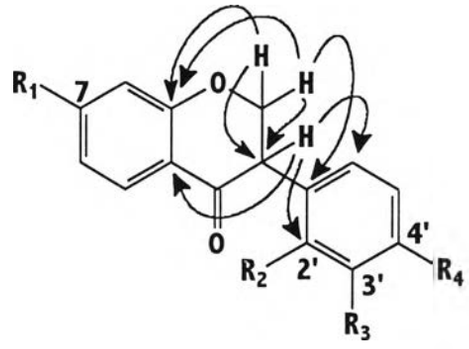
-OH (2)

-OCH_3 (2)

Unambiguous assignments for the ^{1}H and ^{13}C NMR signals in compound 2 were made by combination of the DEPT, NOE DIFF, NOESY, and HMBC spectra. The positions which were substituted by two methoxy groups could be assigned by NOE DIFF. This could also be confirmed through long-range ^{1}H, ^{13}C-coupling deduced from the HMBC spectrum (**Figures 3.14-3.16).** A doublet signal of H-3 (δ_H 4.08 ppm) correlated with C-2' (δ_C 147.4 ppm), C-6' (δ_C 120.8 ppm) and C-10 (δ_C 115.6 ppm). The doublet signal of H-5 (δ_H 7.66 ppm) correlated with C-4 (δ_C 194.4 ppm), C-7 (δ_C 166.4 ppm) and C-9 (δ_C 165.7 ppm). The doublet of doublet signal of H-6 (δ_H 6.50 ppm) correlated with C-8 (δ_C 103.6 ppm) and C-10 (δ_C 115.6 ppm) and the doublet signal of H-8 (δ_H 6.32 ppm) correlated with C-6 (δ_C 111.7 ppm) and C-10 (δ_C 115.6 ppm). The doublet signal of H-5' (δ_H 6.65 ppm) correlated C-1' (δ_C 123.0 ppm) and C-3' (δ_C 140.7 ppm). The doublet signal of H-6' (δ_H 6.53 ppm) correlated with C-2' (δ_C 147.4 ppm), C-4' (δ_C 150.0 ppm) and C-3 (δ_C 49.6 ppm). The unequal proton of methylene at δ 4.41 ppm correlated with C-1'(δ_C 123.0 ppm), C-3(δ_C 49.6 ppm) and C-9(δ_C 165.7 ppm) and at δ 4.50 ppm correlated with C-3(δ_C 49.6 ppm) and C-9(δ_C 165.7 ppm).



HMBC correlation of compound 2

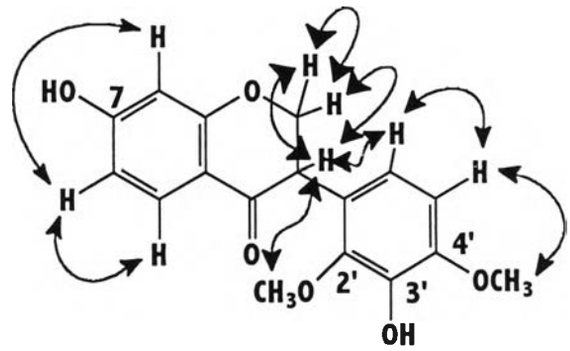


HMBC correlation for compound 2

Table 3.5 ^{1}H, ^{13}C and HMBC spectral data of compound 2 in CD_3OD (500 MHz)

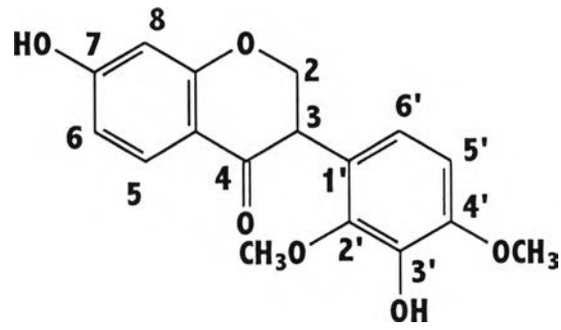
C	δ (ppm)		HMBC (H→ C)
	^{13}C	^{1}H	
2	72.4	4.41	C-3, C-9, C-1'
		4.50	C-3, C-9
3	49.6	4.08	C-10, C-2', C-6'
4	194.4	-	-
5	130.3	7.66	C-4, C-7, C-9
6	111.7	6.50	C-8, C-10
7	166.4	-	-
8	103.6	6.32	C-6, C-10
9	165.7	-	-
10	115.6	-	-
1'	123.0	-	-
2'	147.4	-	-
3'	140.7	-	-
4'	150.0	-	-
5'	107.8	6.65	C-1', C-3'
6'	120.8	6.53	C-3, C-2', C-4'
-OMe	60.4	3.64	C-2'
-OMe	56.6	3.75	C-4'

In an NOE DIFF experiment, irradiation of the proton signal at δ 7.66 only caused enhancement of the proton signal at δ 6.50 (H-6); irradiation of the proton signal at δ 6.50 caused enhancement of the proton signal at δ 7.66 (H-5) and 6.32 (H-8); irradiation of the proton signal at δ 6.32 caused enhancement of the proton signal at δ 6.50 (H-6); irradiation of the proton signal at δ 6.65 caused enhancement of the proton signal at δ 6.53 (H-6') and methoxy protons signal at δ 3.75; irradiation of the proton signal at δ 6.53 caused enhancement of the proton signal at δ 6.65 (H-5') and 4.08 (H-3); irradiation of the proton signal at δ 4.08 caused enhancement of the proton signal at δ 6.53 (H-6') and methoxy protons signal at δ 3.64; irradiation of the proton signal at δ 4.41 caused enhancement proton signal at δ 4.08 (H-3) and 4.50 (H-2) and irradiation of the proton signal at δ 4.50 caused enhancement proton signal at δ 4.08 (H-3) and 4.41 (H-2) (**Figures 3.17-3.23**). The relative proton of compound 2 was also corroborated by the NOESY spectrum (**Figures 3.24-3.25**). The NOE DIFF correlation of compound 2 was displayed as below.

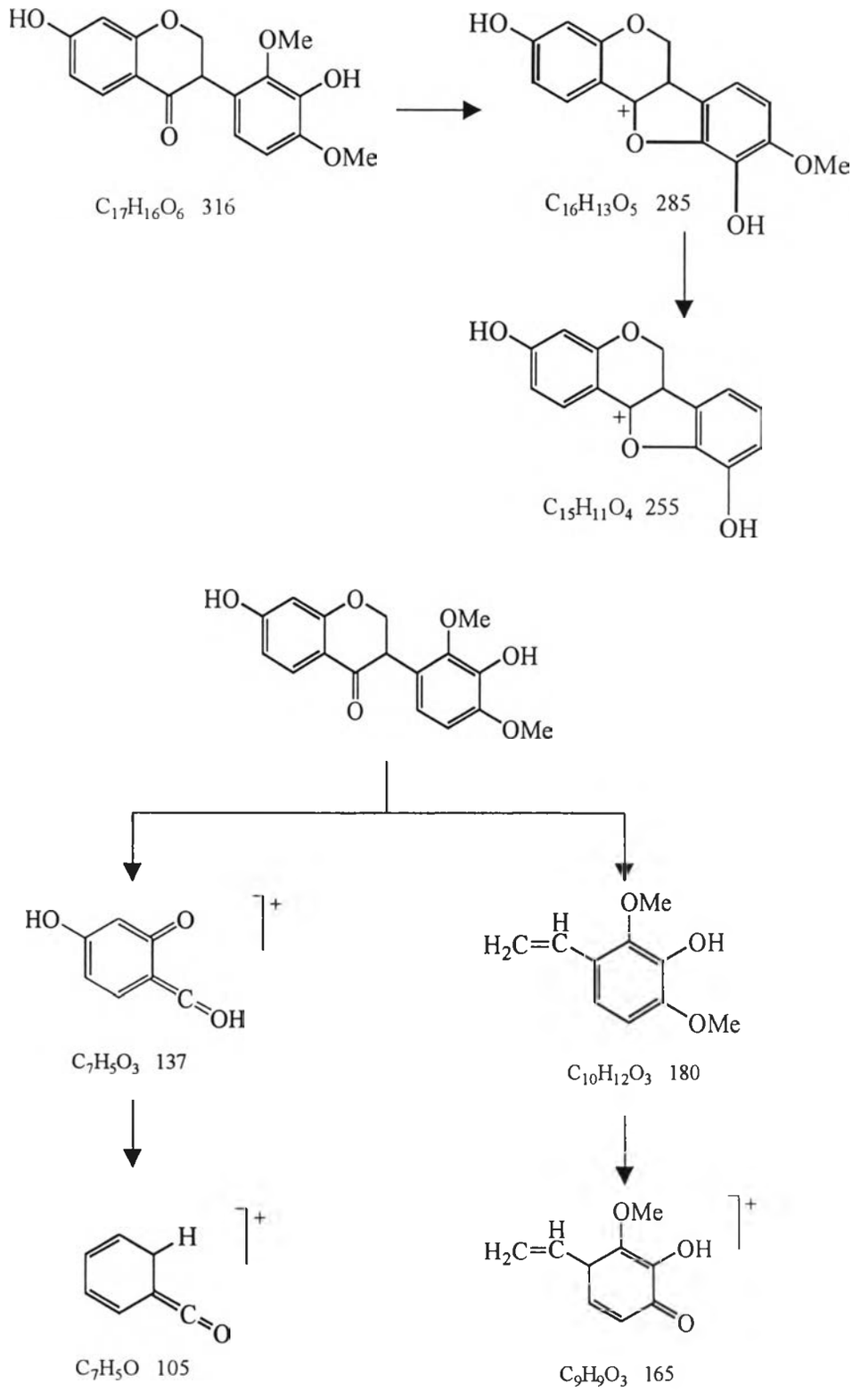


NOE DIFF correlation of compound 2

On the basis of all the spectral data, the structure of compound 2 was established as 7,3'-dihydroxy-2',4'-dimethoxyisoflavanone or violanone.



The structure of compound 2



Scheme 3.2 Possible mass fragmentation patterns of compound 2

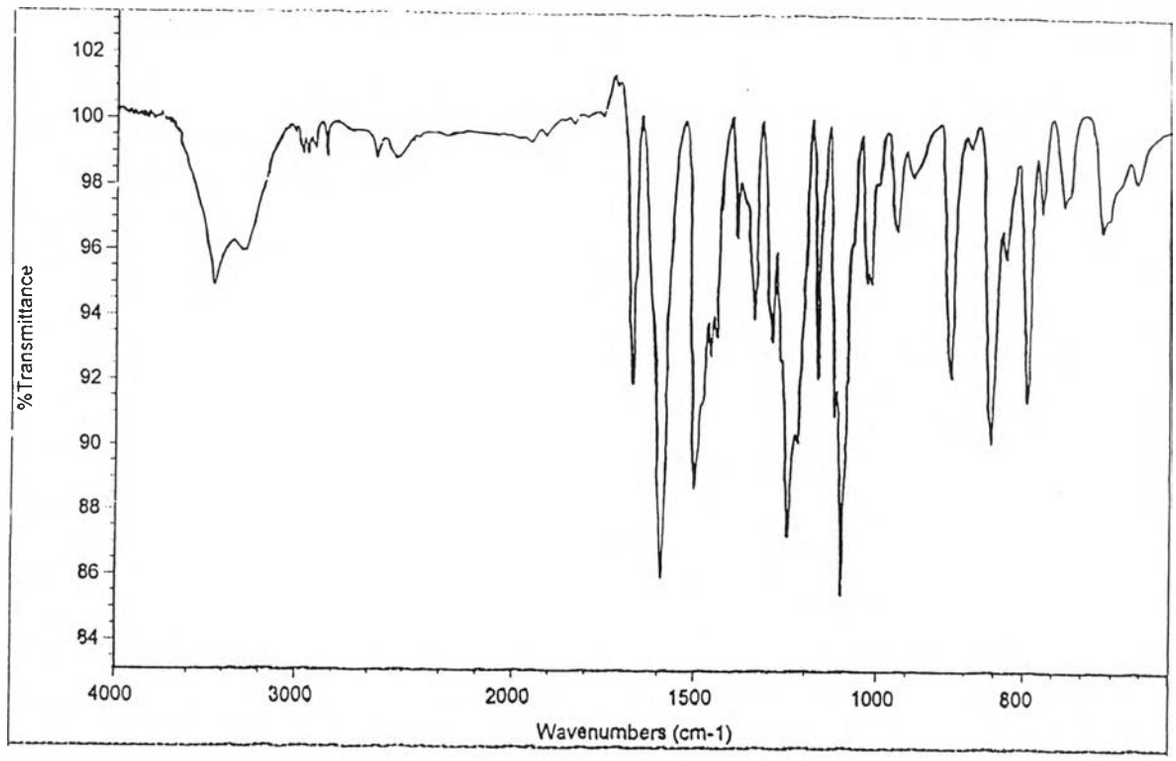


Figure 3.9 IR spectrum of compound 2

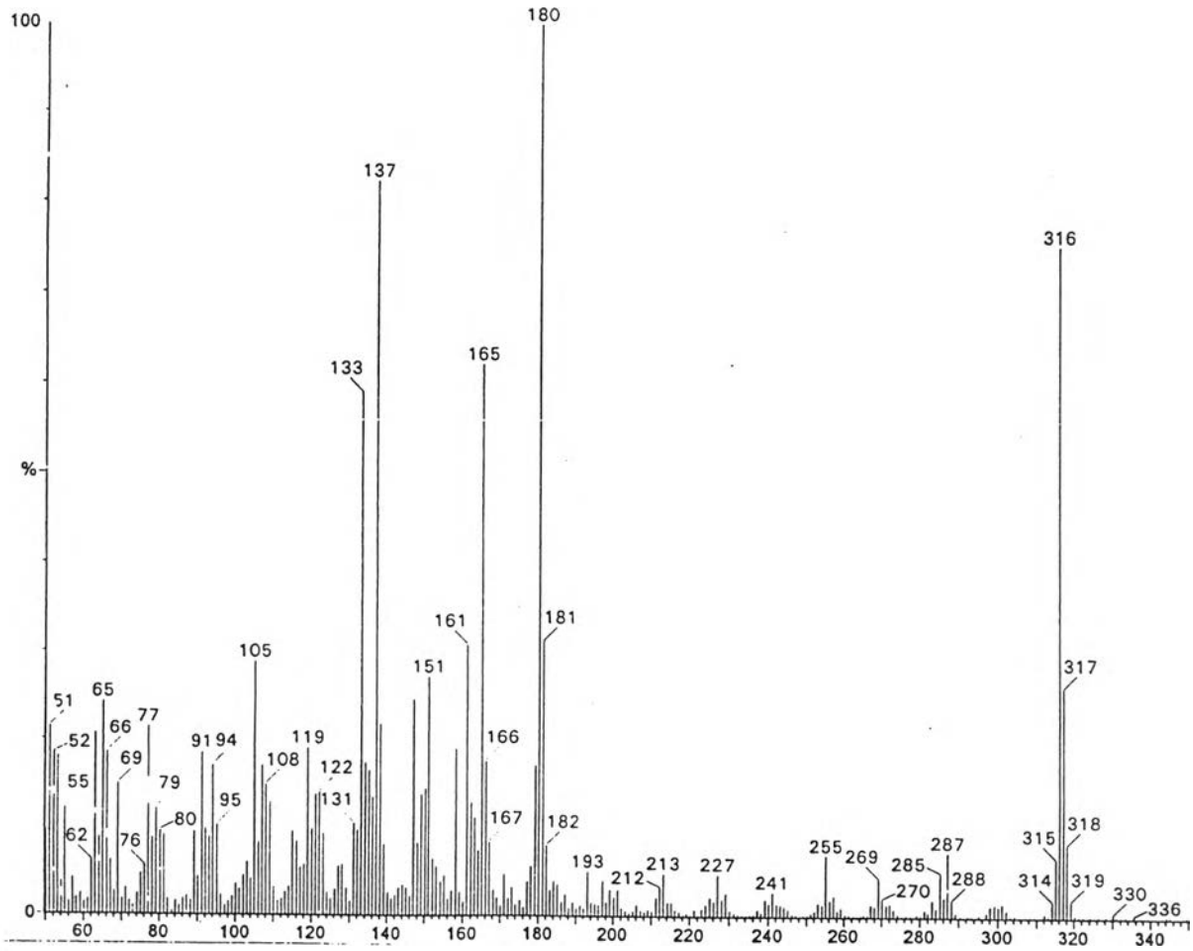


Figure 3.10 Mass spectrum of compound 2

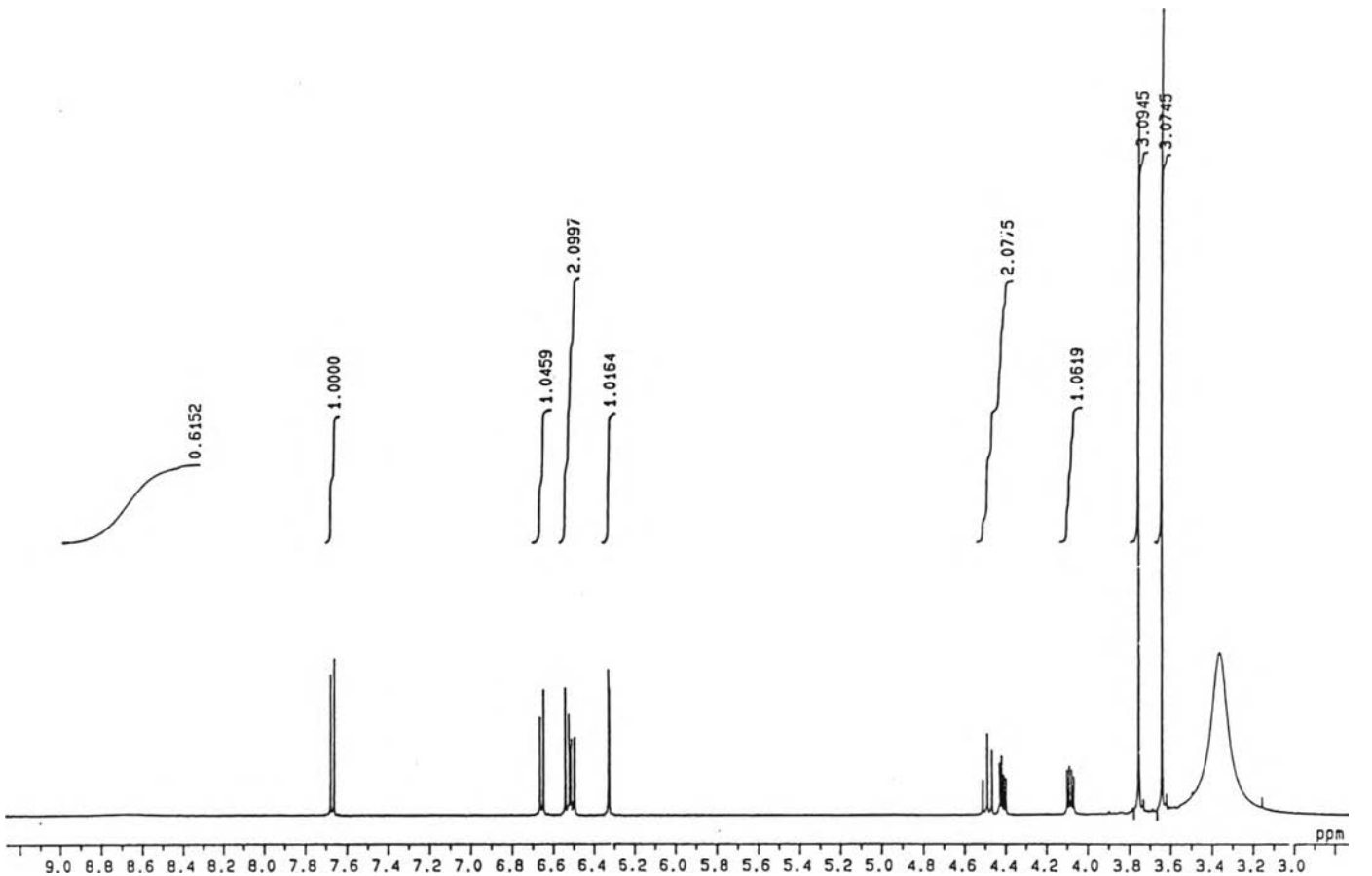


Figure 3.11 ^{1}H-NMR spectrum of compound 2

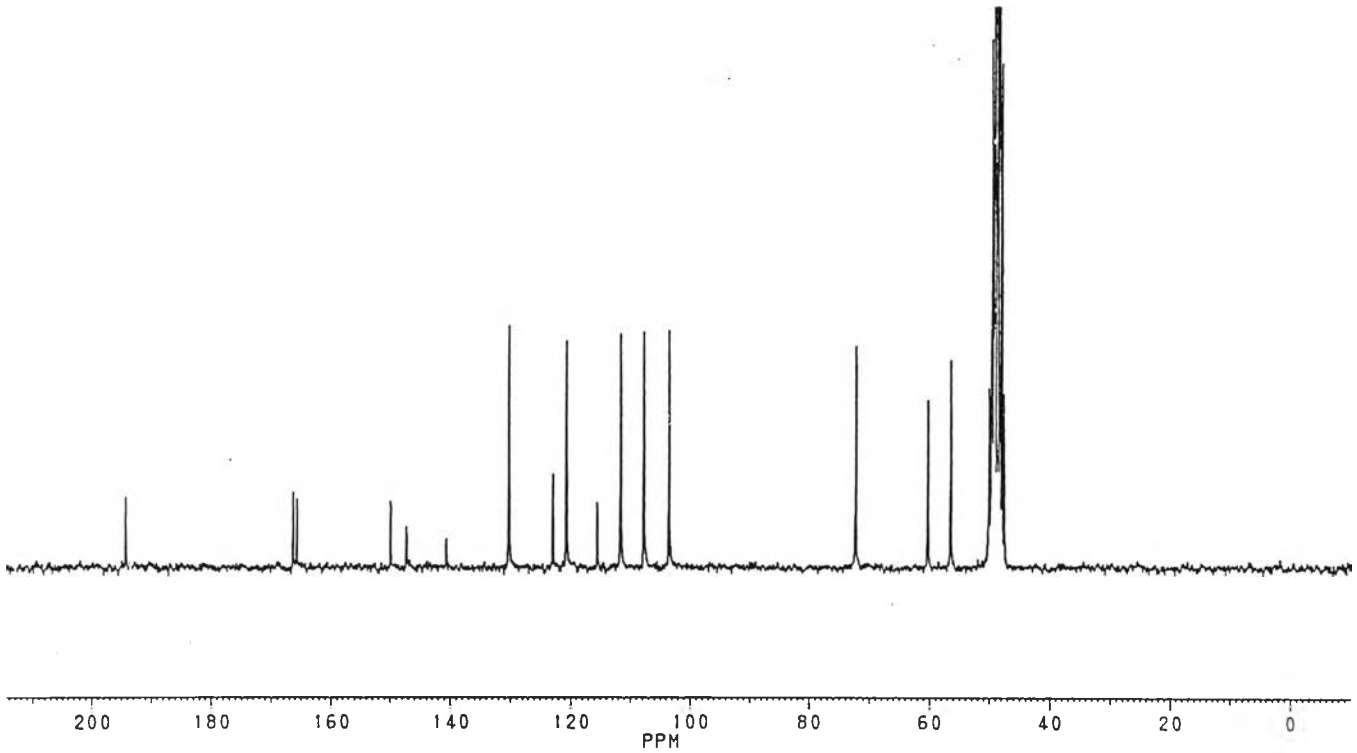


Figure 3.12 ^{13}C-NMR spectrum of compound 2

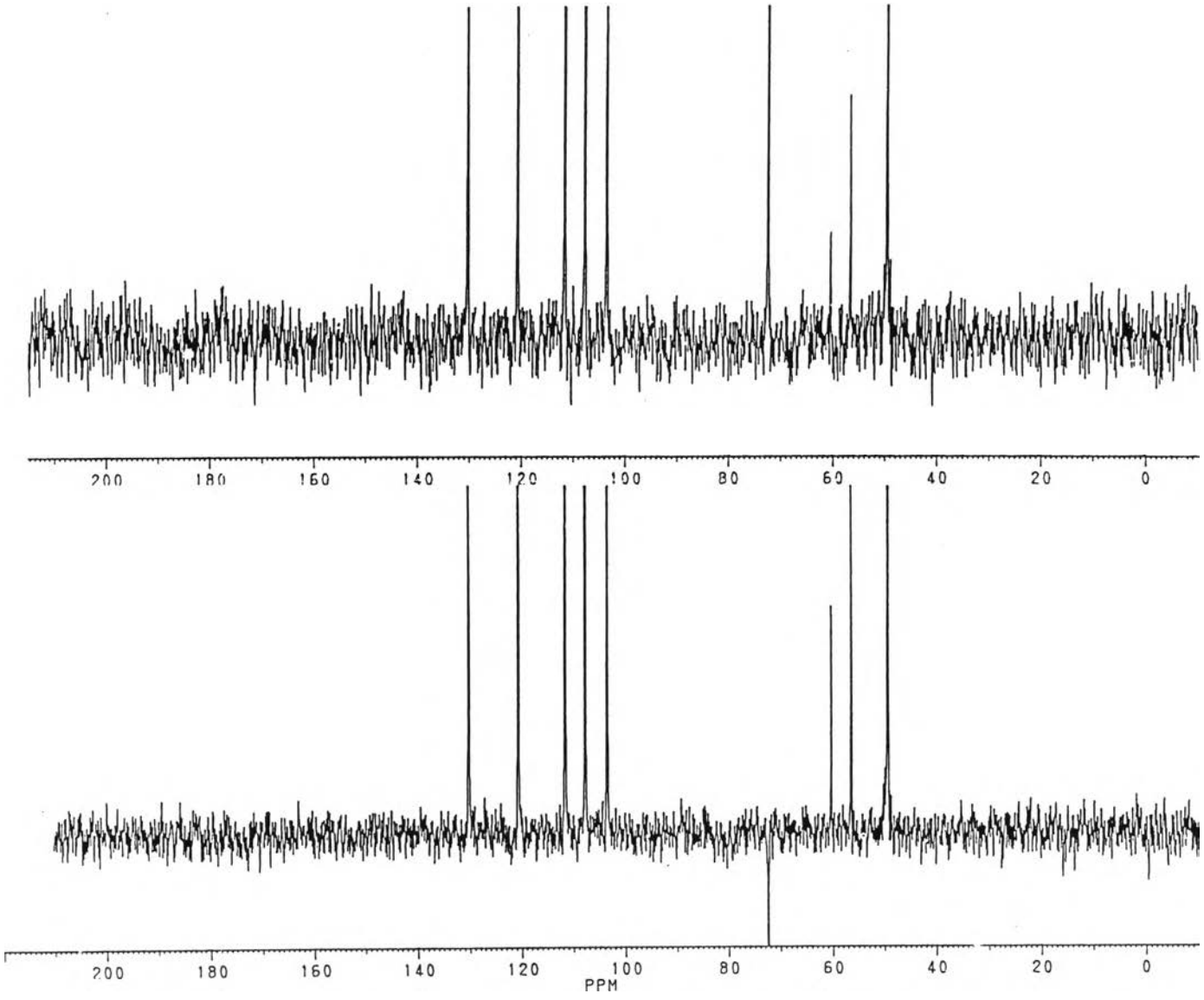


Figure 3.13 DEPT 90 and 135 spectrums of compound 2

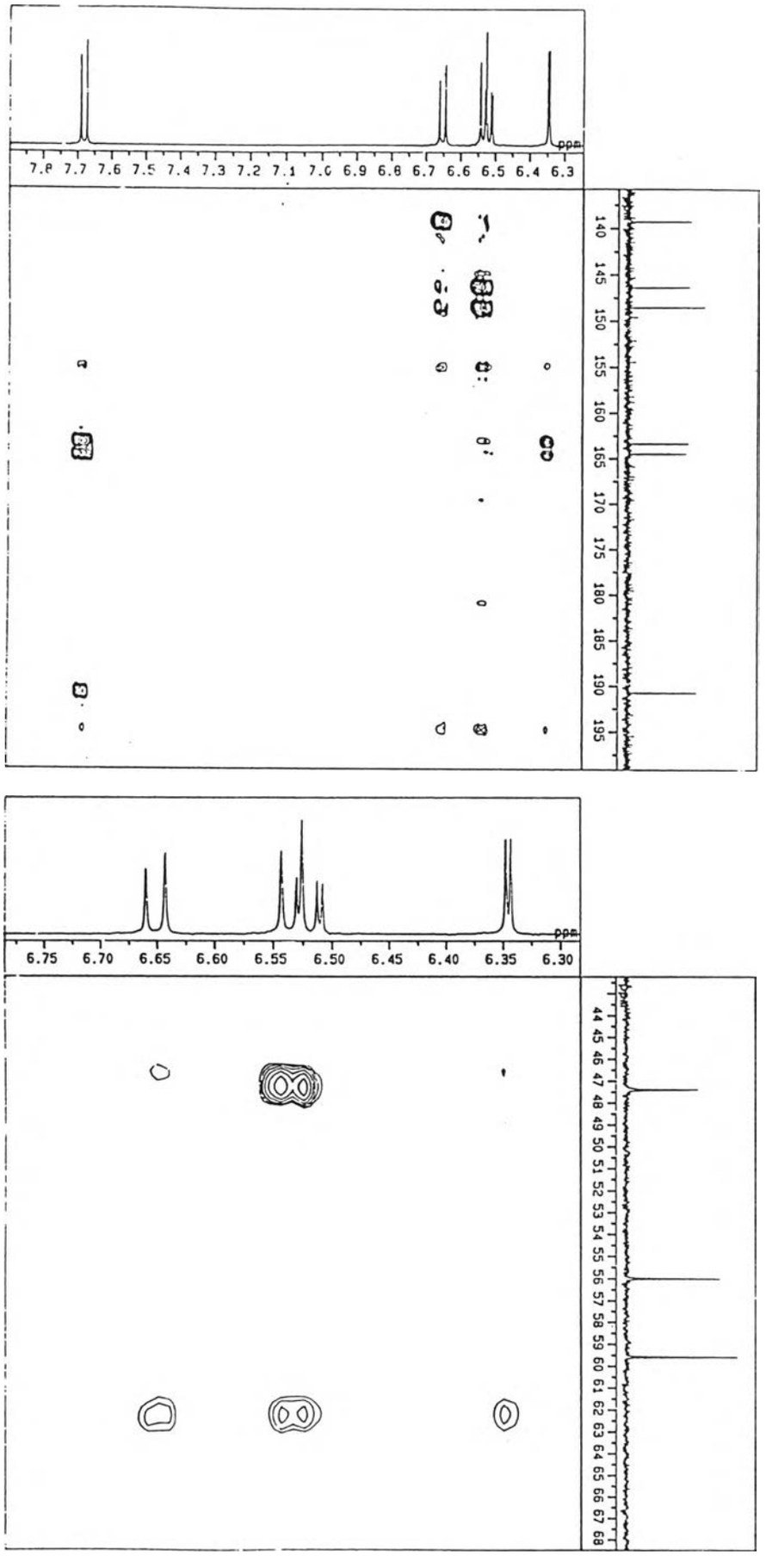


Figure 3.14 HMBC spectrum of compound 2

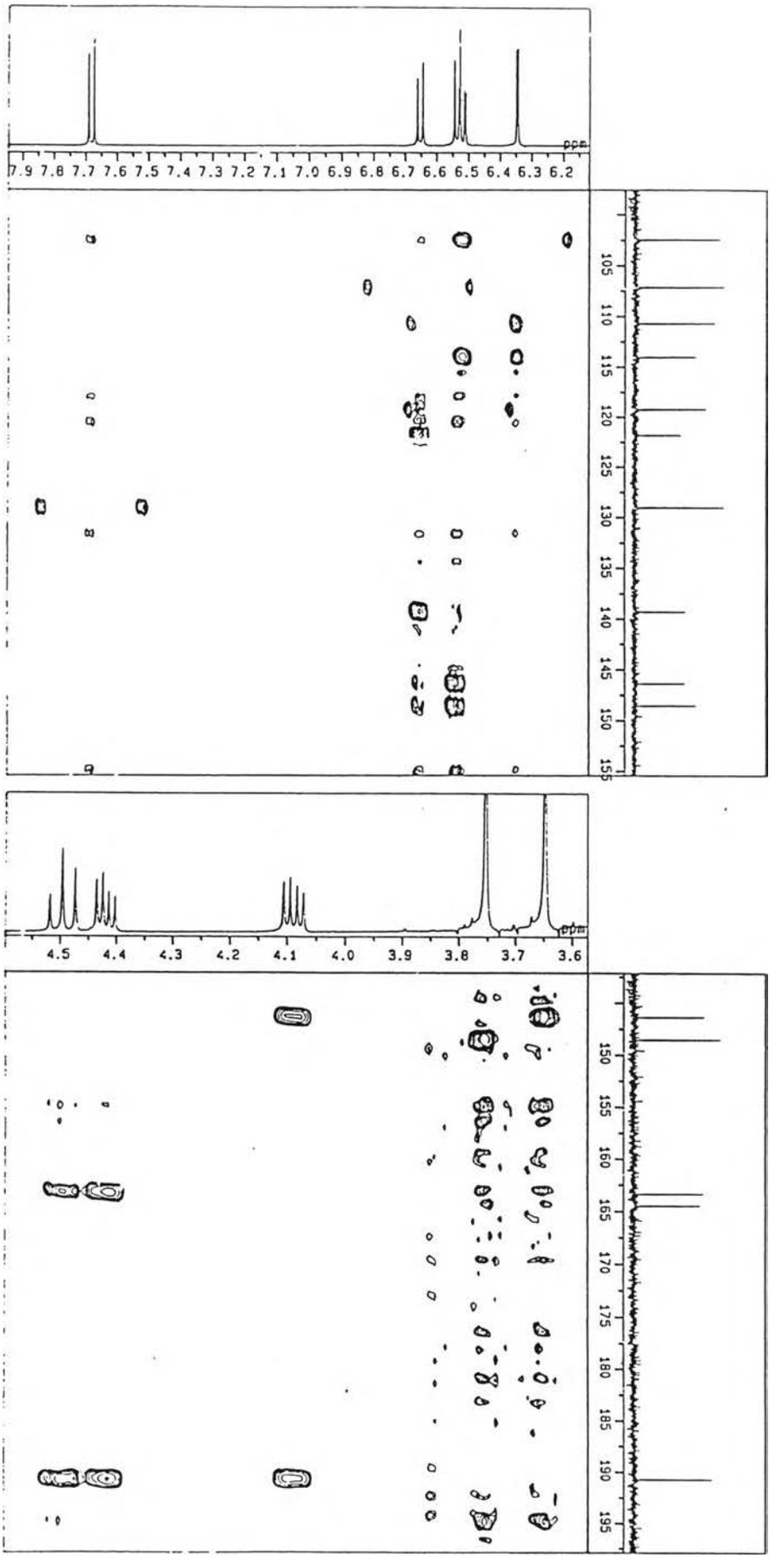


Figure 3.15 HMBC spectrum of compound 2

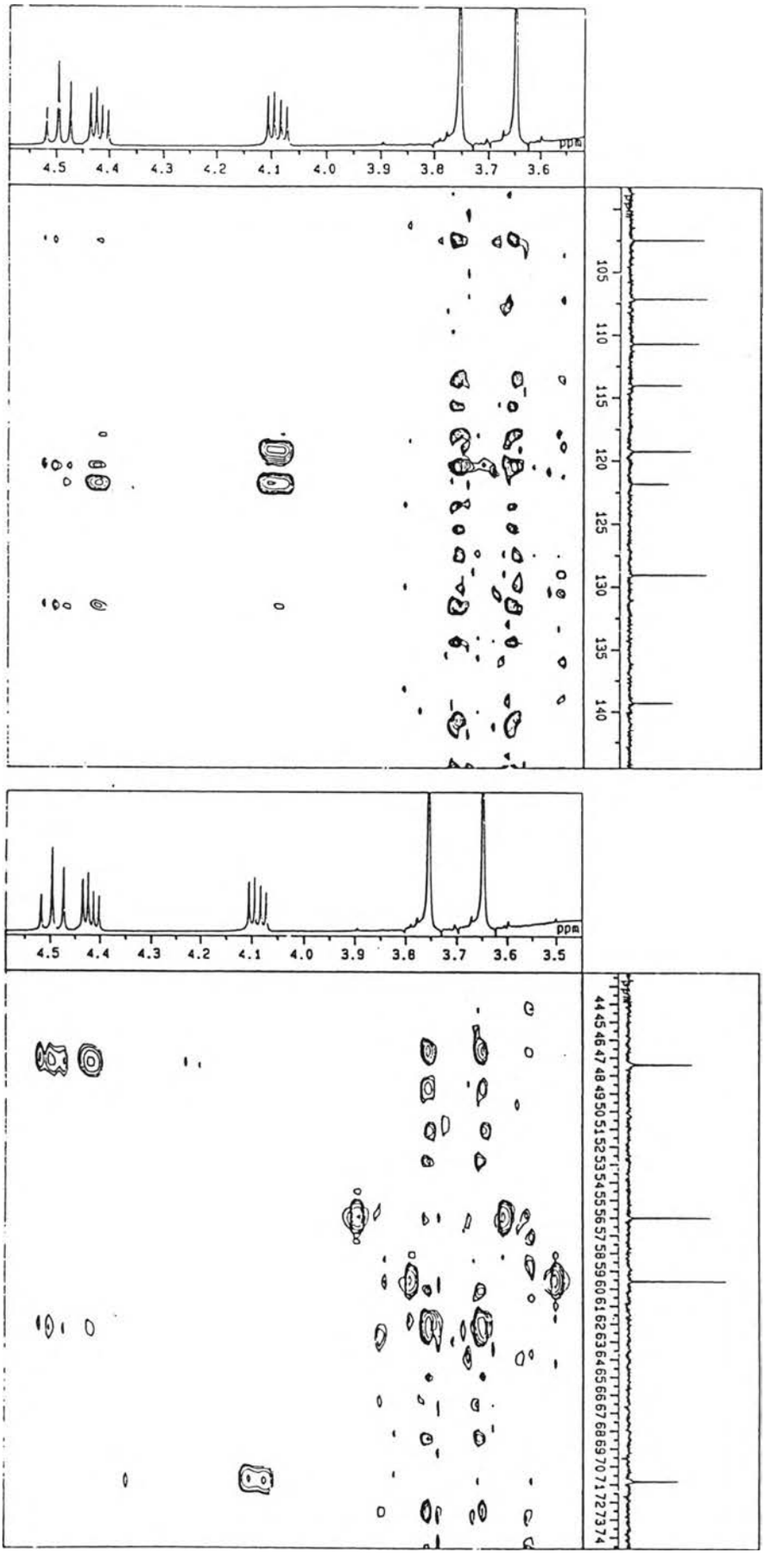


Figure 3.16 HMBC spectrum of compound 2

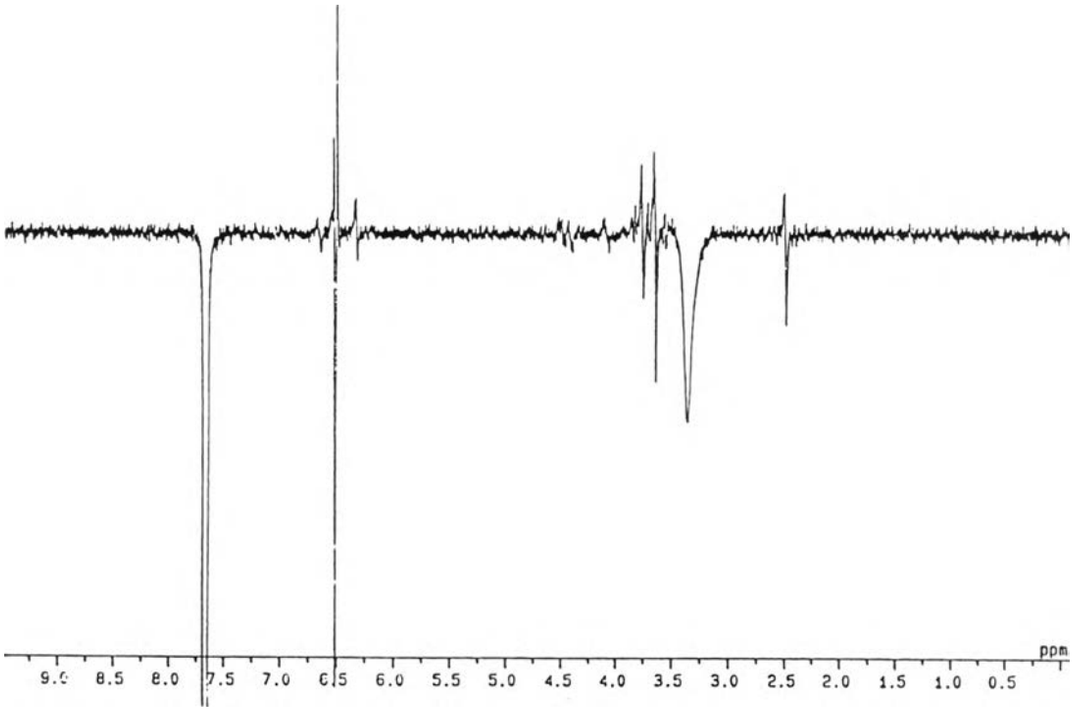


Figure 3.17 NOE DIFF of compound 2 (irradiated at 7.66 ppm)

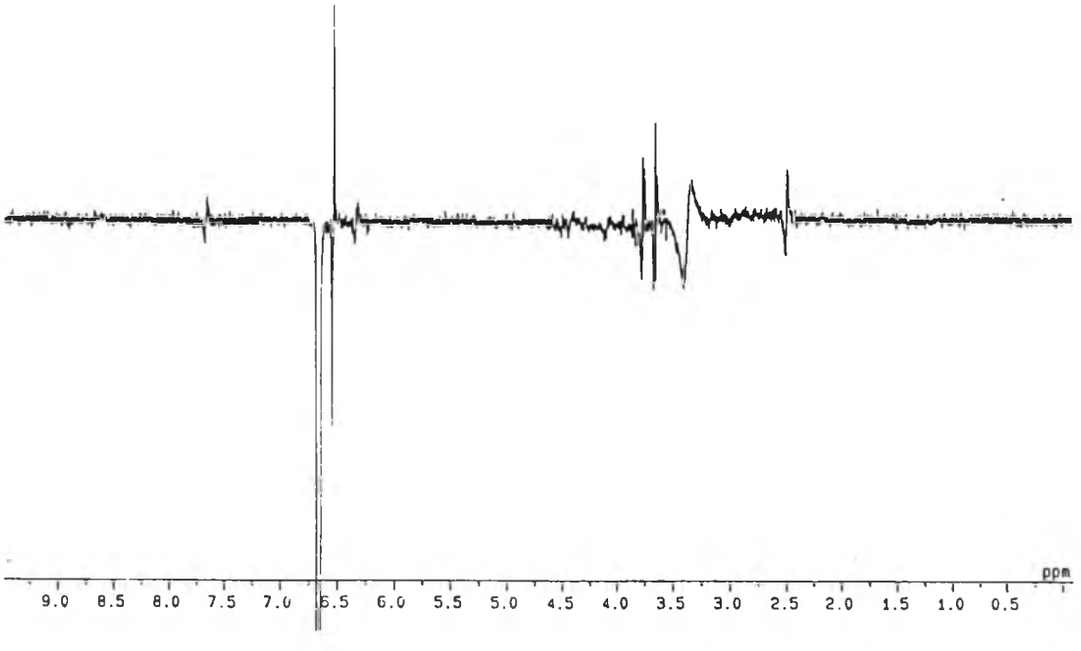


Figure 3.18 NOE DIFF of compound 2 (irradiated at 6.65 ppm)

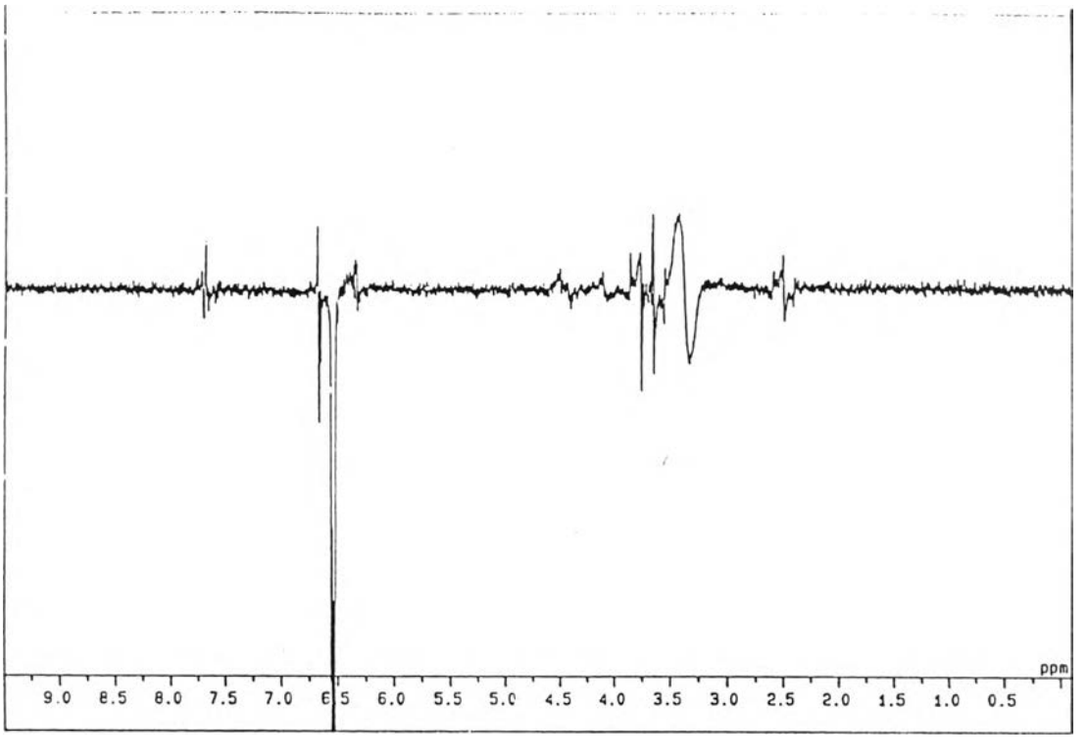


Figure 3.19 NOE DIFF of compound 2 (irradiated at 6.53 ppm)

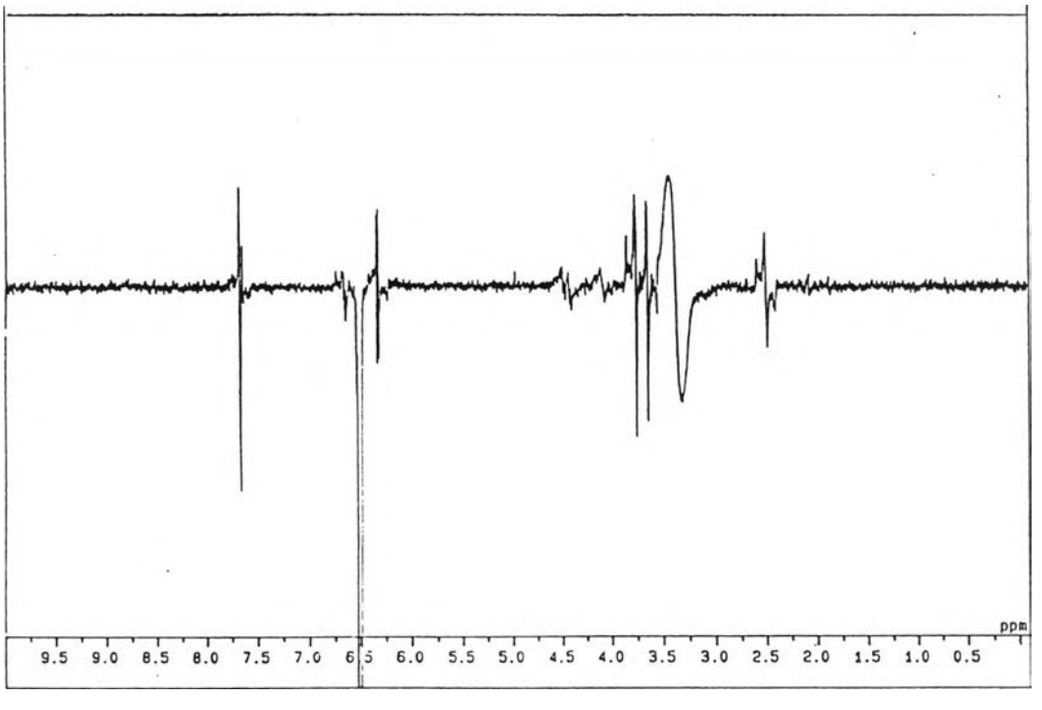


Figure 3.20 NOE DIFF of compound 2 (irradiated at 6.50 ppm)

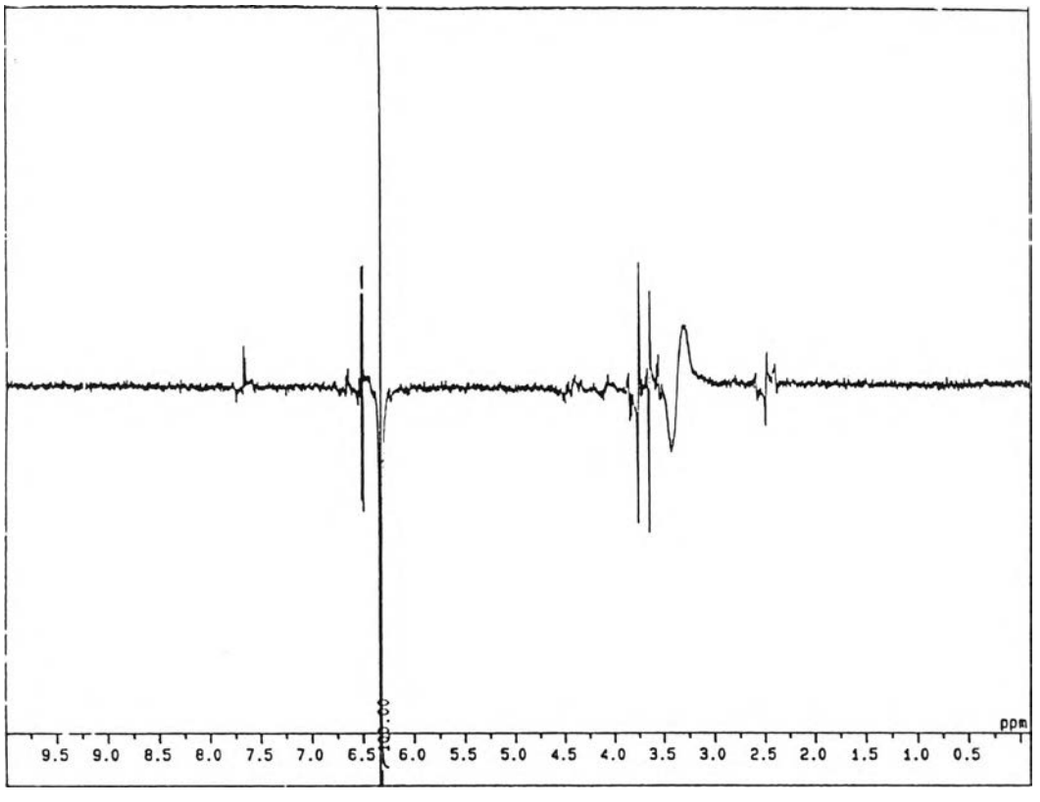


Figure 3.21 NOE DIFF of compound 2 (irradiated at 6.32 ppm)

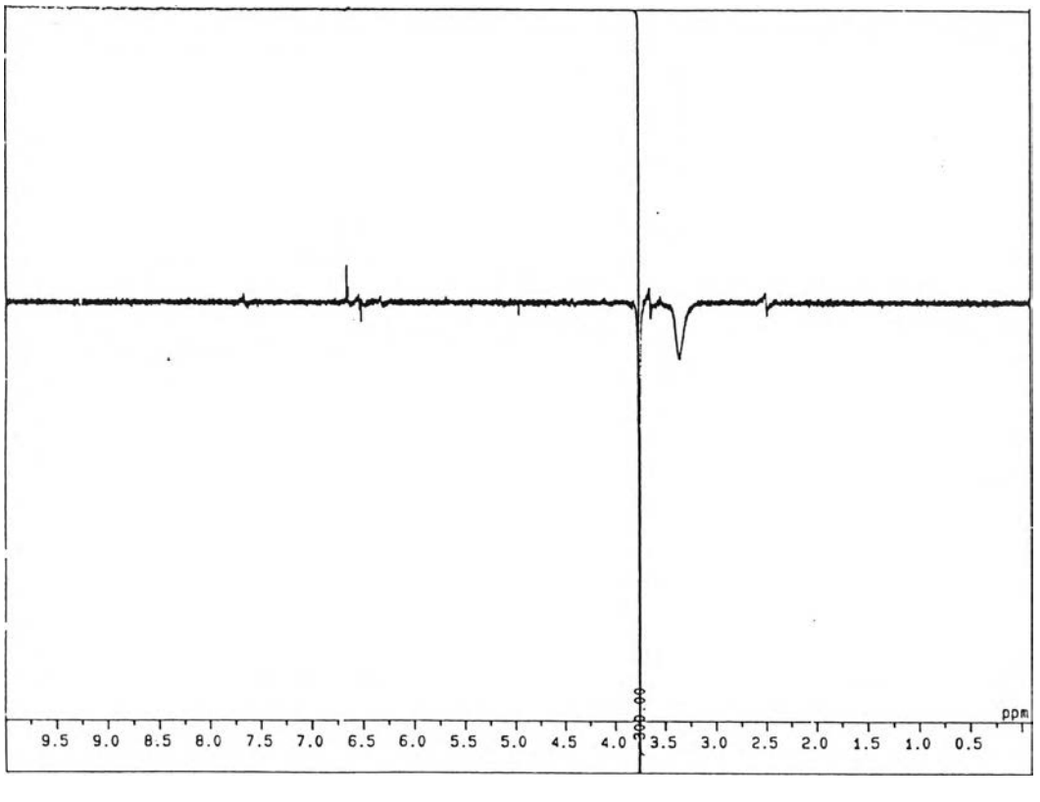


Figure 3.22 NOE DIFF of compound 2 (irradiated at 3.75 ppm)

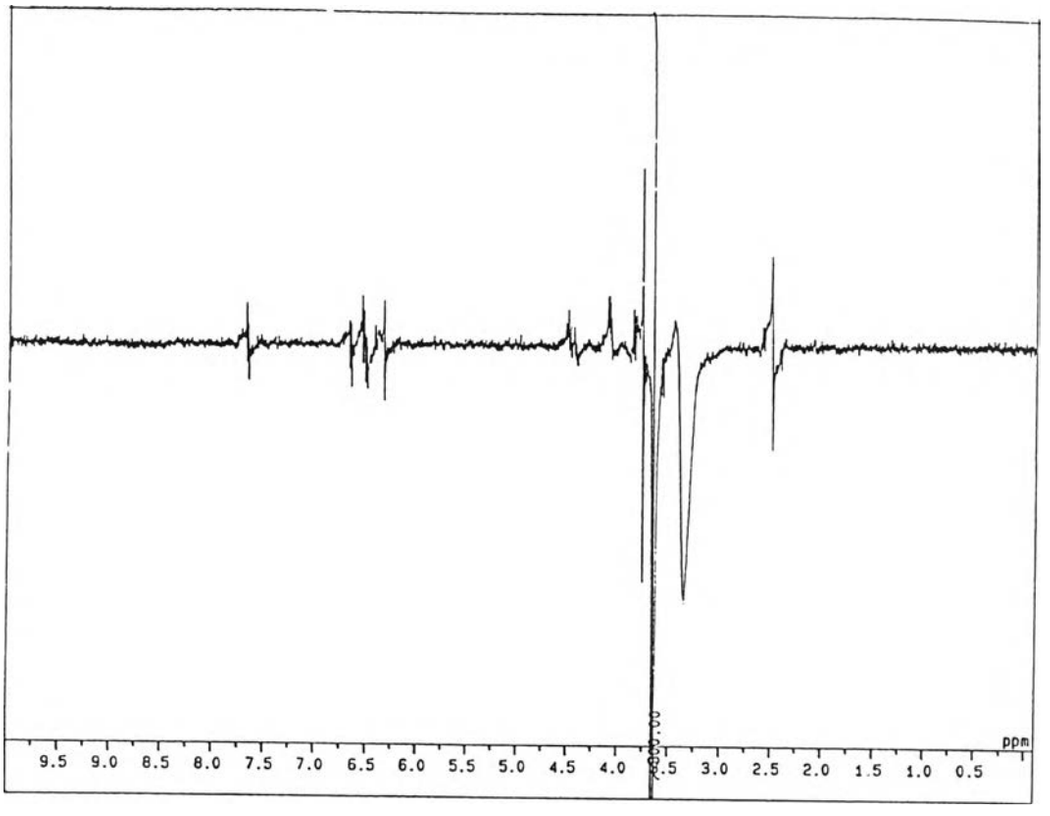


Figure 3.23 NOE DIFF of compound 2 (irradiated at 3.64 ppm)

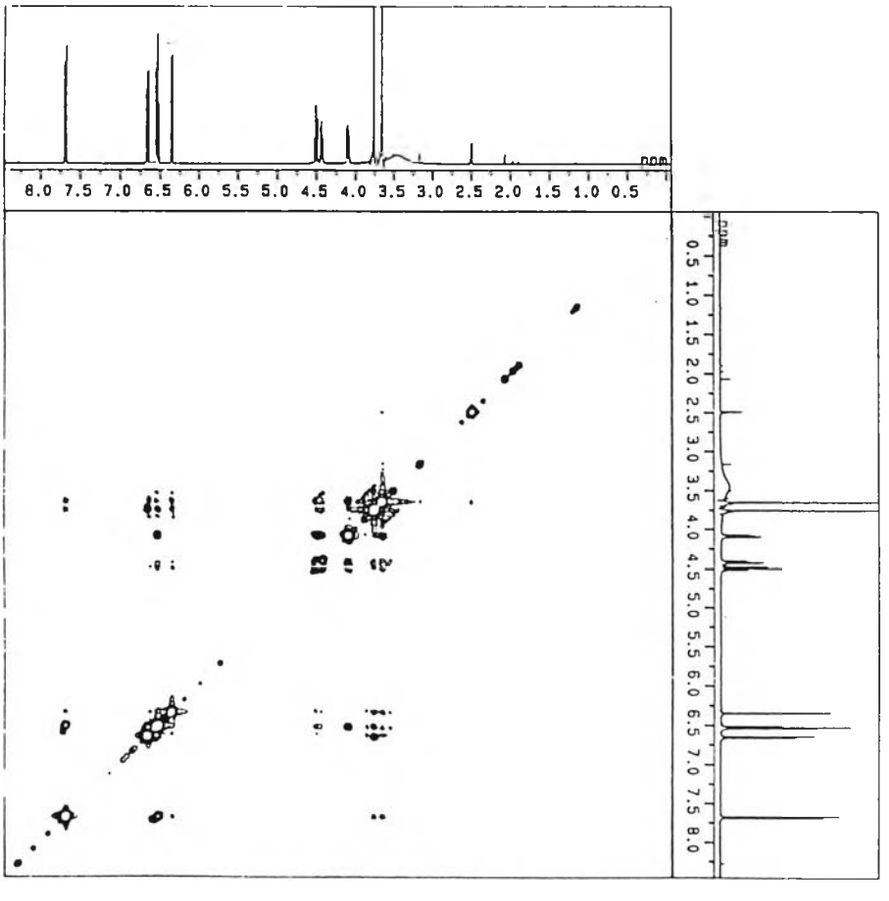


Figure 3.24 NOESY of compound 2

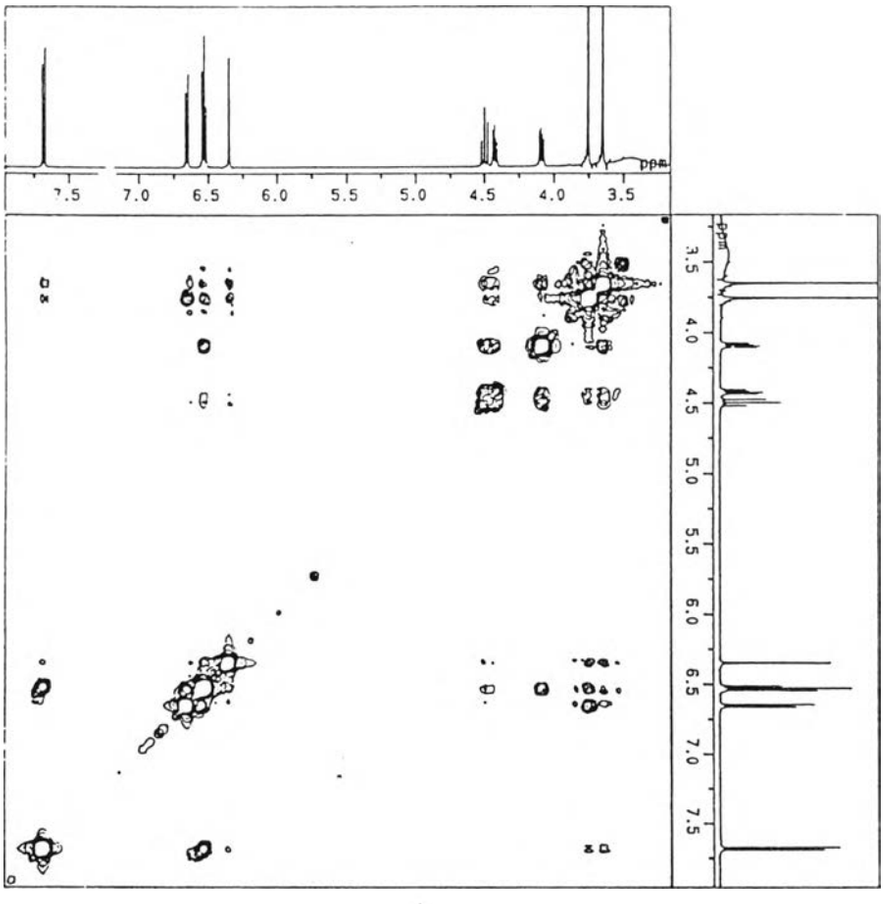


Figure 3.25 NOESY of compound 2

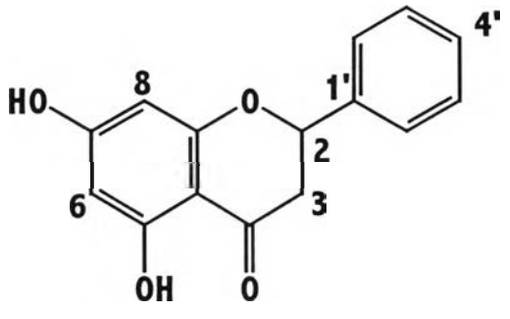
3.2.3 Compound 3

Bright yellow needles (0.4050g) of compound 3 were obtained from dichloromethane crude extract and separated by colunm chromatoghaphy techniques, with m.p. 190-191 °C (lit[24] 192-193 °C)and Rf value 0.595 (SiO_2, EtOAc-CH_2Cl_2, 1:9). The molecular formula of compound 3 was determined as $C_{15}H_{12}O_4$ on the basis of EIMS, ^1H and ^{13}C NMR and showed also a molecular ion peak at m/z 256 [M^+] (**Figure 3.27**)

The IR absorption bands at ν_{max} 3300-3600 (OH stretching vibration), 1648 (C=O stretching vibration), and benzene moieties stretching vibration at 1600, 1481, 1450 and 1422 cm^{-1} (**Figure 3.26**).

^1H NMR (CD_3OD) spectra (**Figure 3.28**), indicated seven aromatic signals at δ 7.45 (2H, *d, J = 7.32 Hz*), 7.35 (3H,*m*), 5.91 (1H, *d, J = 2.44 Hz*), 5.88 (1H, *d, J= 2.13 Hz*), one methine proton at δ 5.38 (1H, *dd, J =12.82, 3.05 Hz*), and one methylene proton at δ 3.03 (1H, *dd, J = 17.09, 12.82 Hz*), and 2.71 (1H, *dd, J = 17.09, 3.05 Hz*) ppm. For ^{13}C NMR (**Figure 3.29**), DEPT 90 and 135 spectra (**Figure 3.30**) exhibited fifteen carbons (flavonoid characteristic); six quarternary carbons at δ 103.3, 140.3, 164.6, 165.4, 168.3, 197.2 (C=O, characteristic of flavone) ppm, eight methine carbons at δ 80.3, 96.2, 97.2, 127.3 (2C), 129.6 (3C) ppm and one methylene carbon at 44.1 ppm.

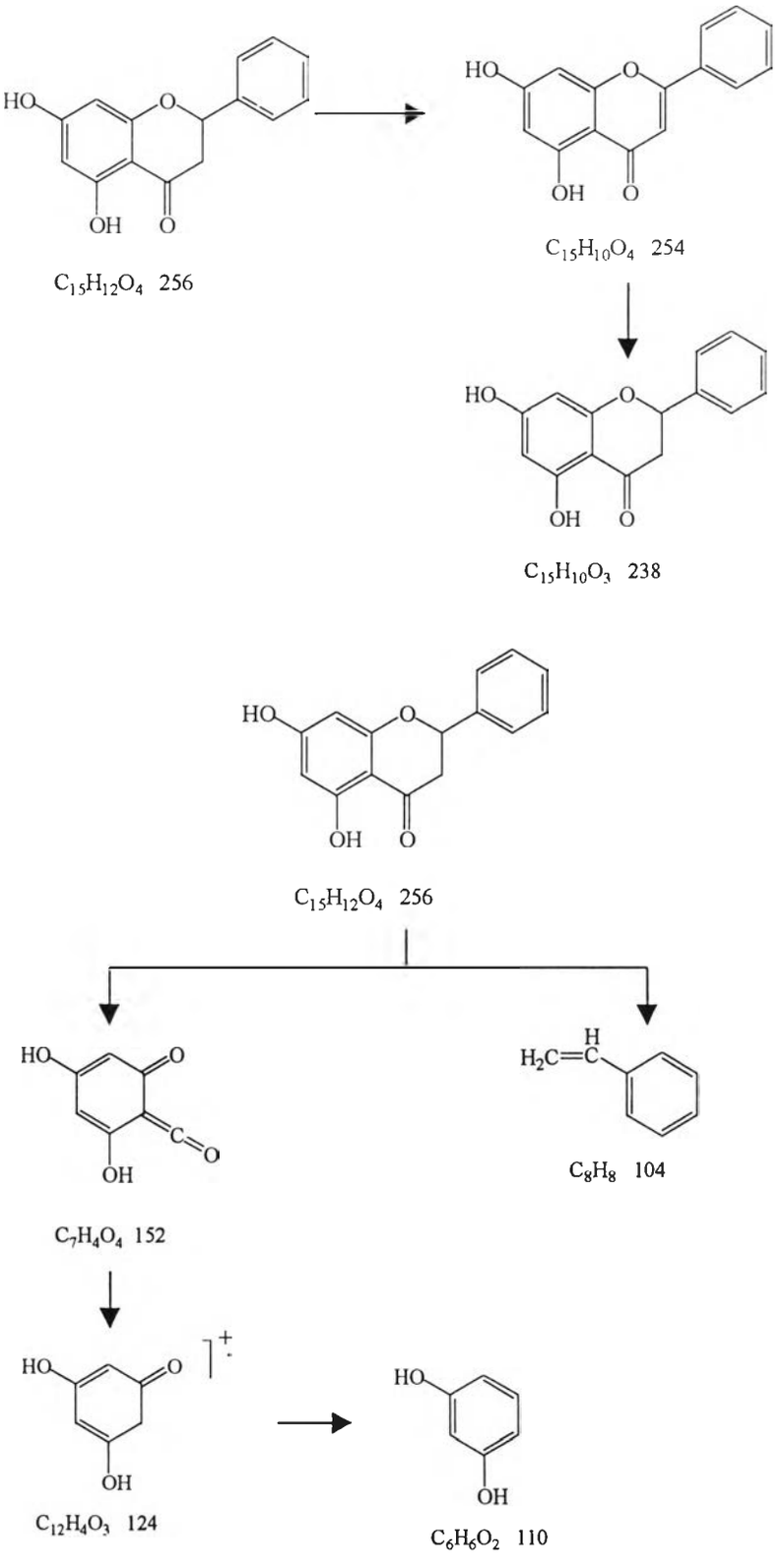
Based on the above spectral characteristic, and a comparison of the ^{13}C NMR spectrum of compound 3 with published data of pinnocembrin, the structure of compound 3 was elucidated as 5,7-dihydroxyflavone or pinnocembrin.



The structure of compound 3

Table 3.6 ^{13}C NMR data of compound 3 compared with pinnocembrin

C	Pinnocembrin[25]	Compound 3
2	78.4	80.3
3	42.2	44.1
4	195.8	197.2
5	163.6	168.3
6	96.1	97.2
7	166.6	165.4
8	95.1	96.2
9	162.7	164.6
10	101.9	103.3
1'	138.0	140.3
2'	126.5	127.3
3'	128.5	129.6
4'	128.5	129.6
5'	128.5	129.6
6'	126.5	127.3



Scheme 3.3 Possible mass fragmentation patterns of compound 3

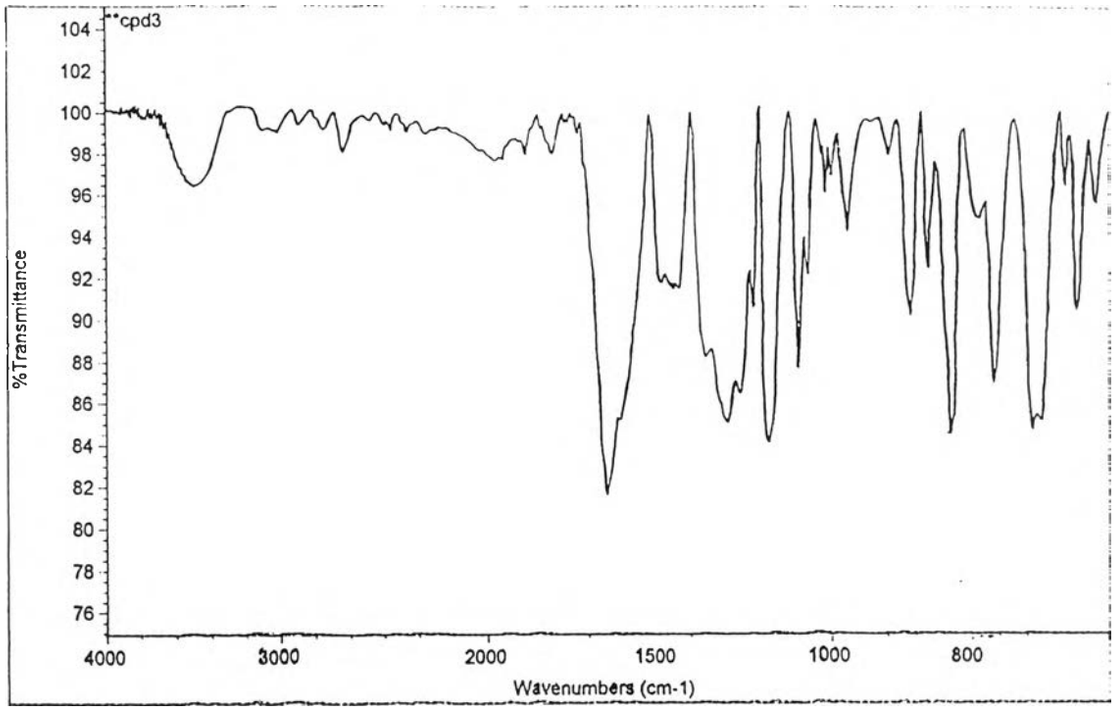


Figure 3.26 IR spectrum of compound 3

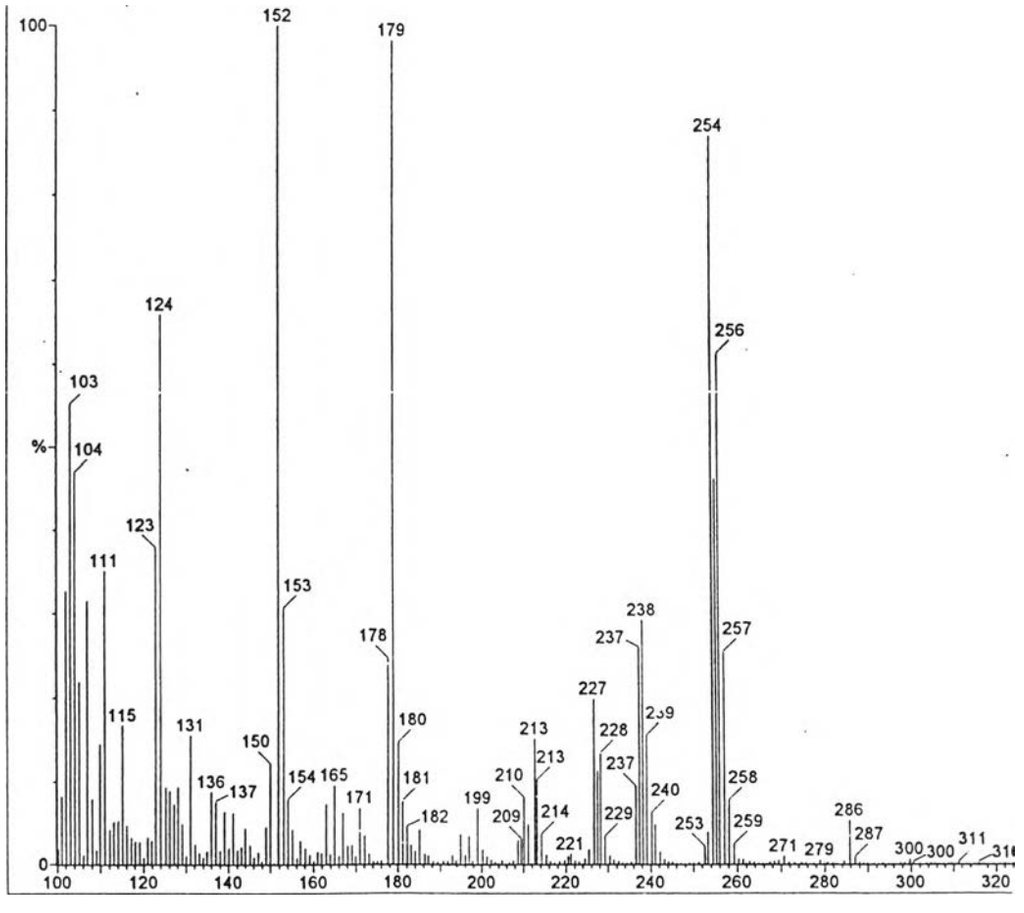


Figure 3.27 Mass spectrum of compound 3

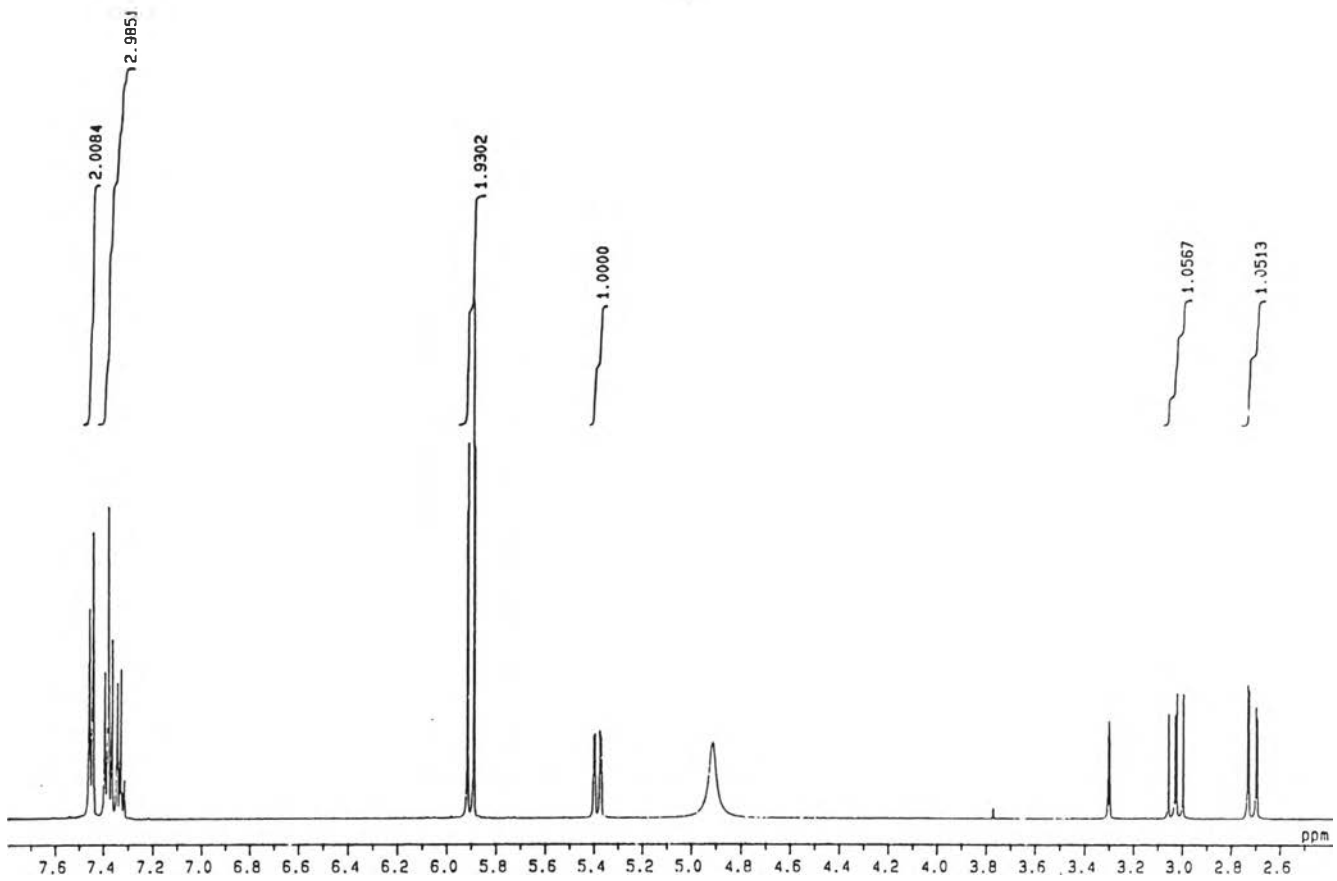


Figure 3.28 ^{1}H-NMR spectrum of compound 3

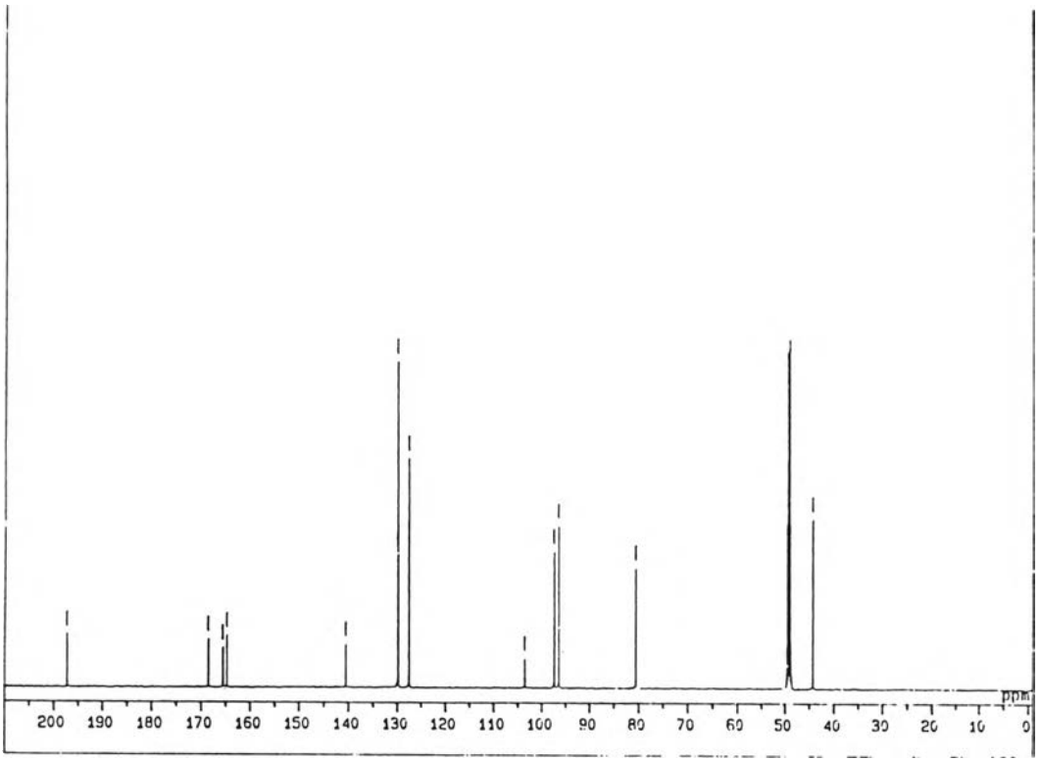


Figure 3.29 ^{13}C-NMR spectrum of compound 3

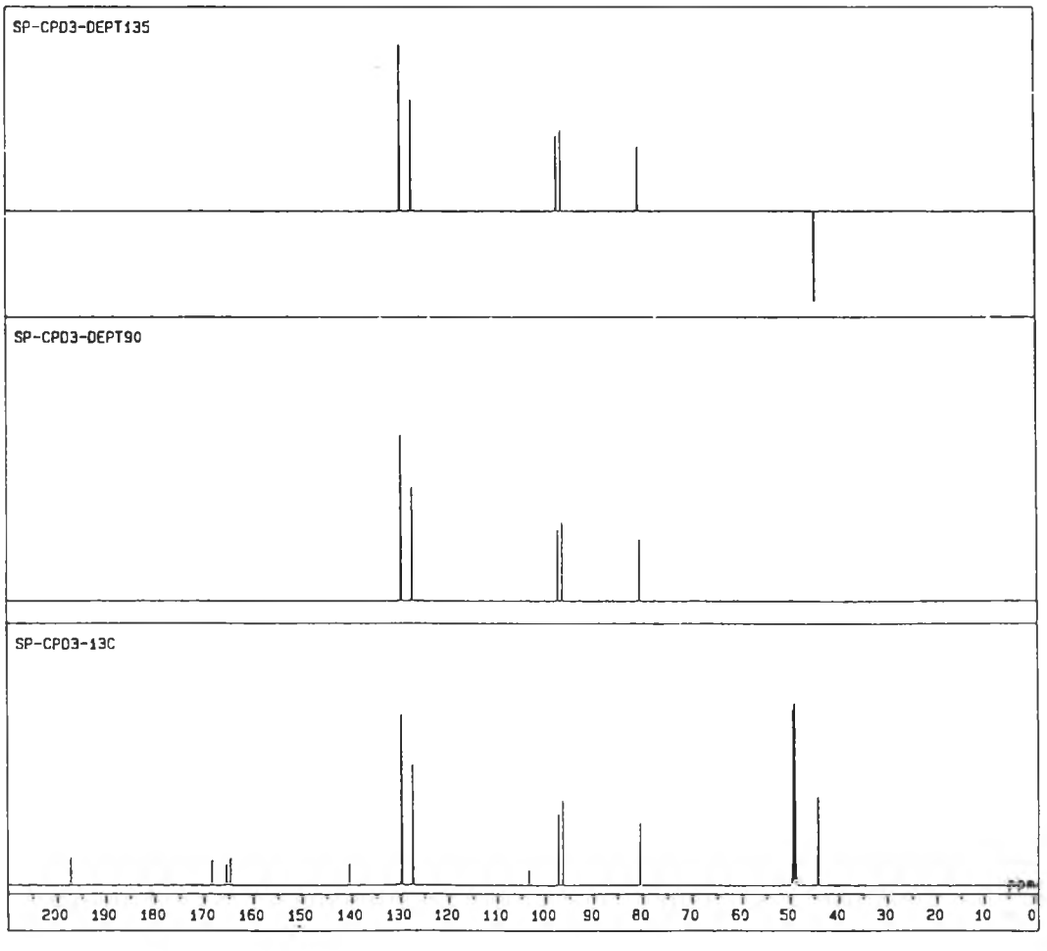


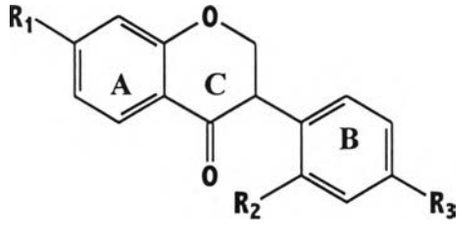
Figure 3.30 DEPT 90 and 135 spectrums of compound 3

3.2.4 Compound 4

Compound 4 was purified by column chromatography of dichloromethane crude extract, white needles (0.1430 g) with m.p. 182-184 °C (lit[26] 182-184 °C) and Rf value 0.39 (SiO_2, EtOAc-CH_2Cl_2, 1:9). Molecular formula was determined to be $C_{17}H_{16}O_5$ on the basis of ^1H, ^{13}C NMR and EIMS data. The EIMS of this compound showed the molecular ion peak at m/z 300 [M^+] (**Figure 3.32**). Its IR spectrum (**Figure 3.31**) indicated the presence of hydroxy groups (3100-3600 cm^{-1}), carbonyl group (1664 cm^{-1}), and benzene ring (1600, 1500 and 1470 cm^{-1}).

The ^1H NMR (CD_3OD) spectrum of compound 4 (**Figure 3.33**) revealed six aromatic proton signals at δ 7.75 (1H, *d*, *J = 8.55 Hz*), 6.98 (1H, *d*, *J = 8.24 Hz*), 6.54 (1H, *d*, *J = 2.44 Hz*), 6.49 (1H, *dd*, *J = 2.44, 2.13 Hz*), 6.46 (1H, *dd*, *J = 2.44, 2.44 Hz*), 6.32 (1H, *d*, *J = 2.13 Hz*) ppm, and one methylene proton at δ 4.53 (1H, *d*, *J = 8.6 Hz*), 4.38 (1H, *dd*, *J = 5.50, 5.49 Hz*) and two methyl protons in methoxy group at δ 3.76 and 3.74 ppm.

The ^{13}C NMR spectra (**Figure 3.34**) appeared seventeen carbons signals of fifteen skeleton and two substitute groups (characteristic of flavonoid); two methyl carbons of methoxy groups at δ 55.7 (OCH_3) and 55.9 (OCH_3) ppm. The DEPT 90, and 135 spectra (**Figures 3.35-3.36**) also showed as seven quarternary carbons at δ 115.7 (C-10), 117.3 (C-1'), 159.8 (C-2'), 162.1 (C-4'), 165.7 (C-9), 166.4 (C-7), 194.3 (C-4, C=O, characteristic of the isoflavanone) ppm, seven methine carbons at δ 132.0 (C-6'), 130.5 (C-5), 111.7 (C-6), 105.9 (C-5'), 103.6 (C-8), 99.9 (C-3'), 49.0 (C-3) ppm, one methylene carbon at δ 72.0 ppm. All of the above data suggested the possibility of a flavonoid skeleton of isoflavanone type. Therefore, the data and comparison with ^1H, ^{13}C NMR of known isoflavanone suggests that the molecule of this compound must have the basic skeleton shown below:



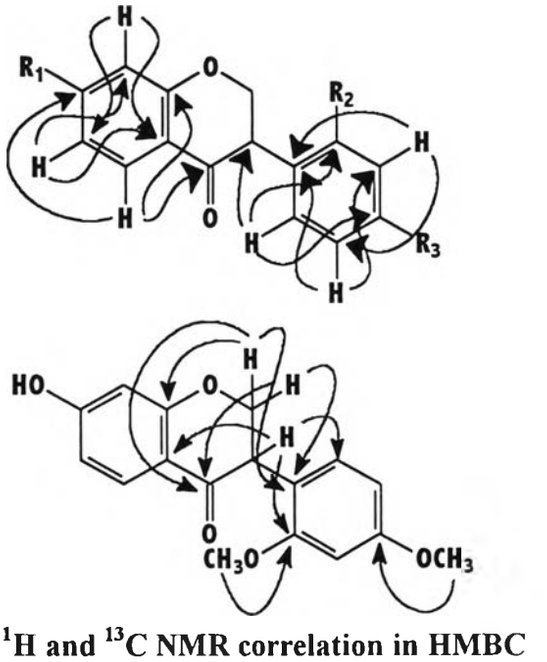
Substituents:

-OH (1)

-OCH_3 (2)

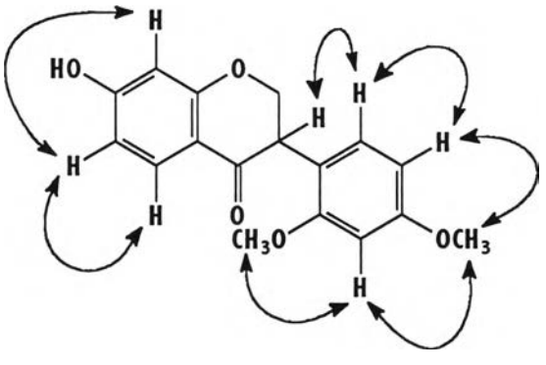
One Bond Correlation (HMQC) data revealed that the proton at δ 4.14 ppm was attached to the carbon at δ 49.0 ppm, the proton at δ 7.75 ppm was attached to the carbon at δ 130.5 ppm, the proton at δ 6.49 ppm was attached to the carbon at δ 111.7 ppm, the proton at δ 6.32 ppm was attached to the carbon at δ 103.6 ppm, the proton at δ 6.54 ppm was joined with the carbon at 99.9 ppm, the proton at δ 6.46 ppm was joined with the carbon at δ 105.9 ppm, and the proton at δ 6.98 ppm was joined with the carbon at δ 132.0 ppm. The methoxys groups at δ 3.74 and 3.76 ppm were joined with the carbon at δ 55.9 and 55.7 ppm, respectively (**Figures 3.37-3.38**).

The relative of this compound between protons and carbons were confirmed by HMBC (**Figures 3.39-3.42**) and NOE DIFF experiments. The aromatic in ring A doublet signal at δ 6.32 ppm could be assigned for H-8, which correlated with C-6 (δ_C 111.7 ppm) and C-10 (δ_C 115.7 ppm). The doublet of doublet signal at δ 6.49 ppm was designated for H-6 ,which correlated with C-8 (δ_C 103.6 ppm) and C-10 (δ_C 115.6 ppm). The remaining aromatic proton at δ 7.75 ppm (H-5) was also correlated to C-7 (δ_C 166.4 ppm) and C-9 (δ_C 165.7 ppm). In ring B proton signal at δ 6.54 ppm could be approved for H-3',which relatived with C-1' and C-5', proton signal at δ 6.46 ppm (H-5') was correlated to C-1' and C-3', and the remaining proton at δ 6.98 ppm (H-6') was correlated with C-2' and C-4'. In compound 4, a HMBC correlation was also evident between methoxy protons (δ_H 3.74) with C-2' (δ_C 159.8) and (δ_H 3.76) with C-4' (δ_C 162.1).



^{1}H and ^{13}C NMR correlation in HMBC

The NOE DIFF experiments were used to obtain addition information. Irradiation of the proton at δ 7.75 ppm caused enhancement of the signal at δ 6.49 ppm and irradiation of the proton at δ 6.98 ppm caused enhancement of the signal at δ 6.49 and 4.14 ppm. Irradiation of the proton at δ 6.55 ppm caused enhancement of the signal at δ 3.74 and 3.76 ppm, irradiation of the proton at δ 6.49 ppm caused enhancement of the signal at δ 7.75 ppm. Irradiation of the proton at δ 6.47 ppm caused enhancement of the signal at δ 3.76 ppm, irradiation in methoxy protons at δ 3.76 ppm caused enhancement of the signal at δ 6.55 and 6.47 ppm and other methoxy protons at δ 3.74 ppm caused enhancement of the signal at δ 6.55 ppm (**Figures 43-49**).



The NOE DIFF correlation of compound 4

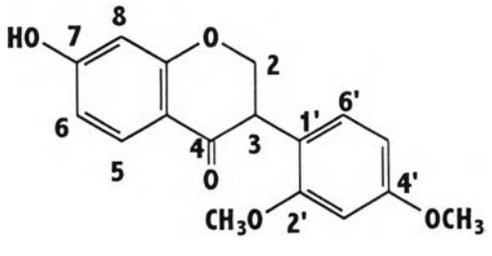
Table 3.7 ^{1}H, ^{13}C and HMBC spectral data of compound 4 in CD_3OD (500 MHz)

C	δ (ppm)		HMBC (H→ C)
	^{13}C	^{1}H	
2	72.0	4.38	C-3, C-4, C-9, C-1'
		4.53	C-3, C-4, C-9
3	49.0	4.15	C-2, C-4, C-1', C-2', C-6'
4	194.3	-	-
5	130.5	7.75	C-4, C-7, C-9
6	111.7	6.49	C-8, C-10
7	166.4	-	-
8	103.6	6.32	C-6, C-10
9	165.7	-	-
10	115.7	-	-
1'	117.3	-	-
2'	159.8	-	-
3'	99.9	6.54	C-1', C-5'
4'	162.1	-	-
5'	105.9	6.46	C-1', C-3'
6'	132.0	6.98	C-2', C-4'
-OMe	55.9	3.64	C-2'
-OMe	55.7	3.75	C-4'

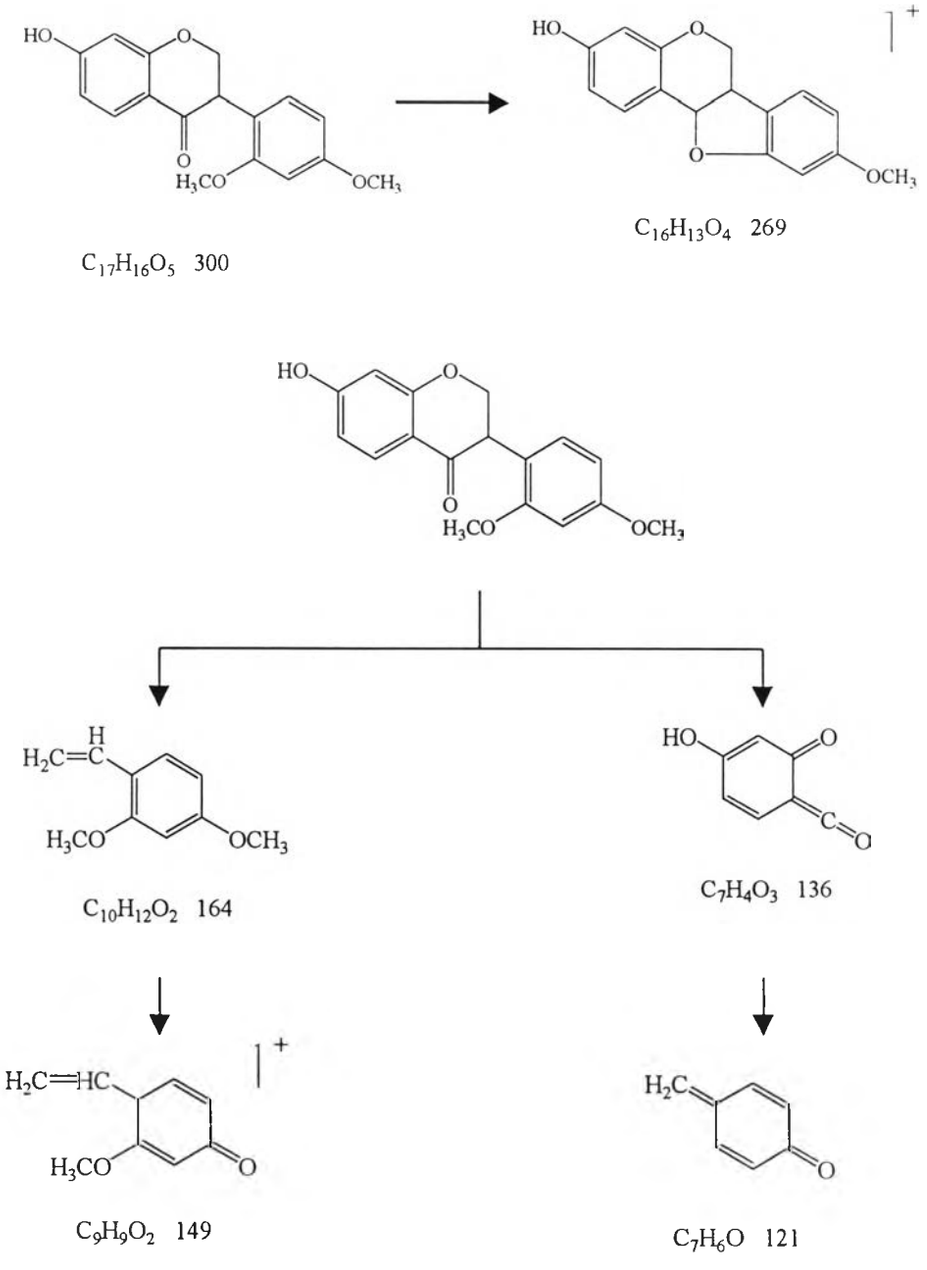
Table 3.8 ^{13}C NMR data of compound 4 compared with sativanone

Position of Carbon	Chemical shift (ppm)	
	Compound 4	Sativanone[26]
2	72.0	71.8
3	49.0	48.0
4	194.3	191.6
5	130.5	131.5
6	111.7	103.4
7	166.4	165.1
8	103.6	99.7
9	165.7	164.7
10	115.7	115.8
1'	117.3	117.4
2'	159.8	159.5
3'	99.9	111.2
4'	162.1	161.5
5'	105.9	105.7
6'	132.0	130.0
2'-OCH_3	55.9	55.9
4'-OCH_3	55.7	55.6

Analysis of all the information above permitted compound 4 to be identified as 7-hydroxy-2',4'-dimethoxyisoflavanone or sativanone.



The structure of compound 4



Scheme 3.4 Possible mass fragmentation patterns of compound 4

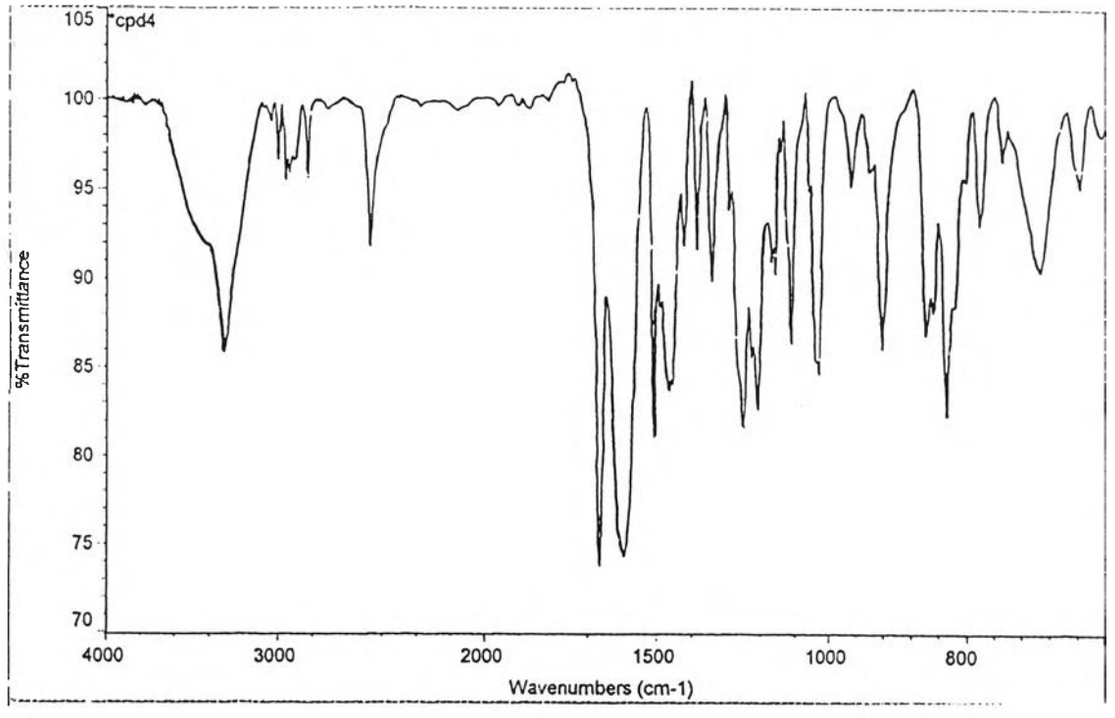


Figure 3.31 IR spectrum of compound 4

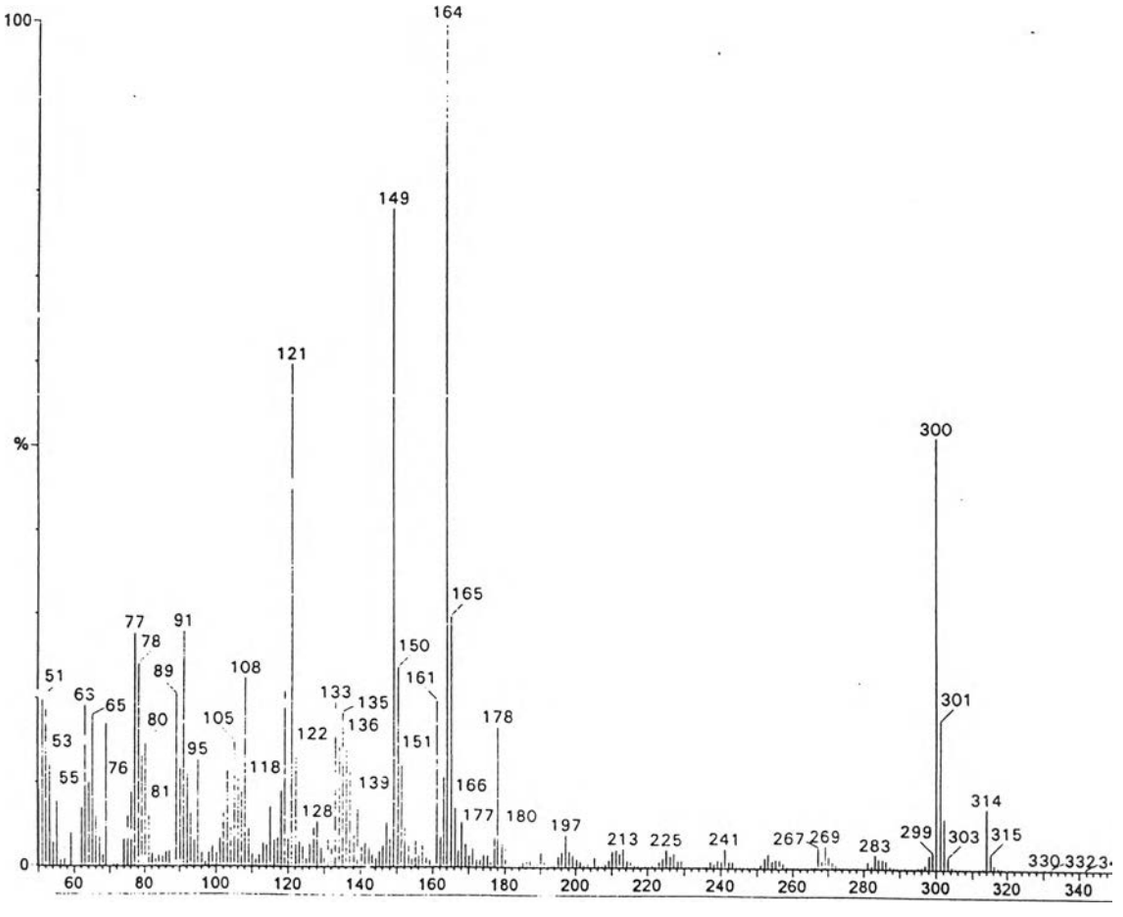


Figure 3.32 Mass spectrum of compound 4

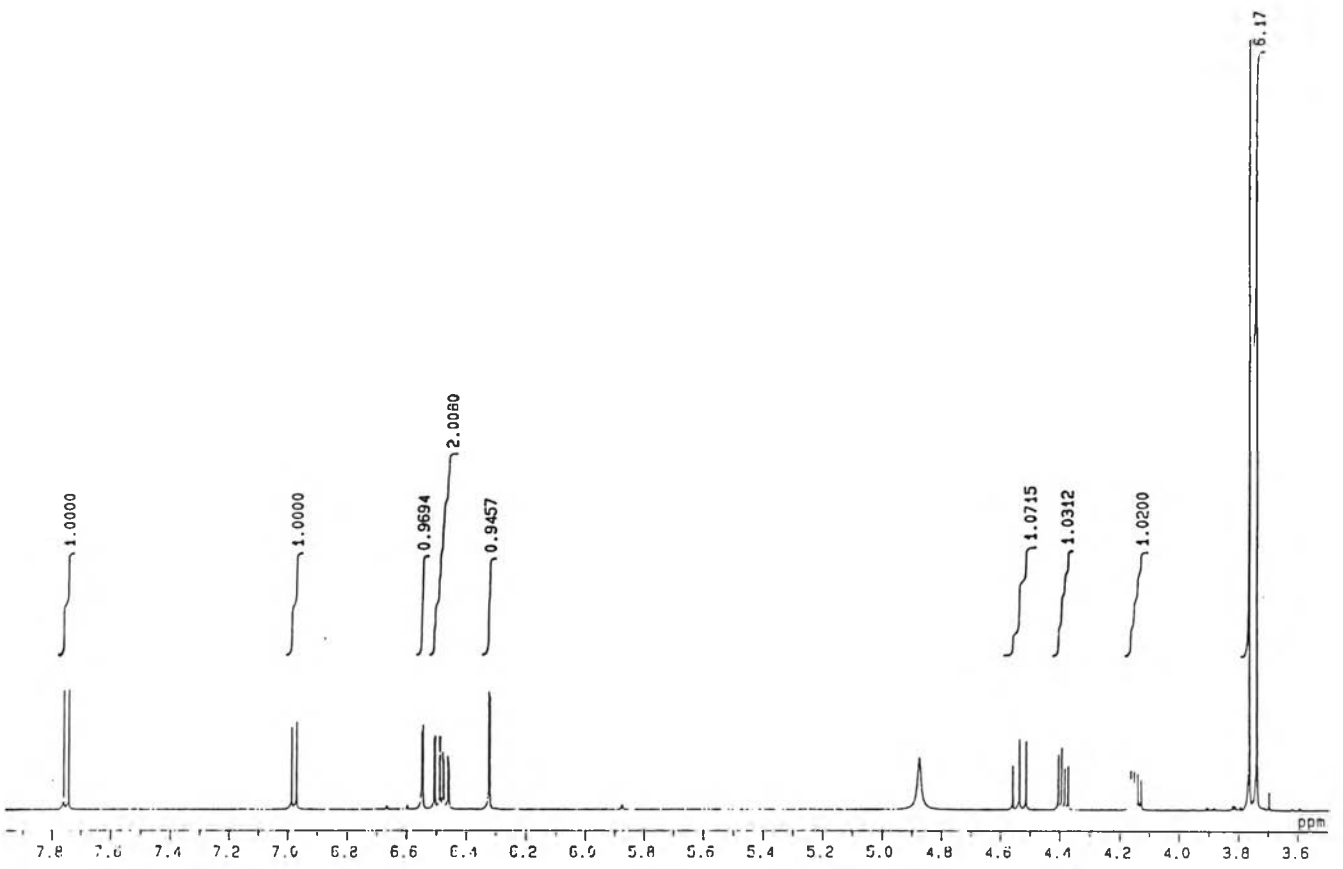


Figure 3.33 ^{1}H-NMR spectrum of compound 4

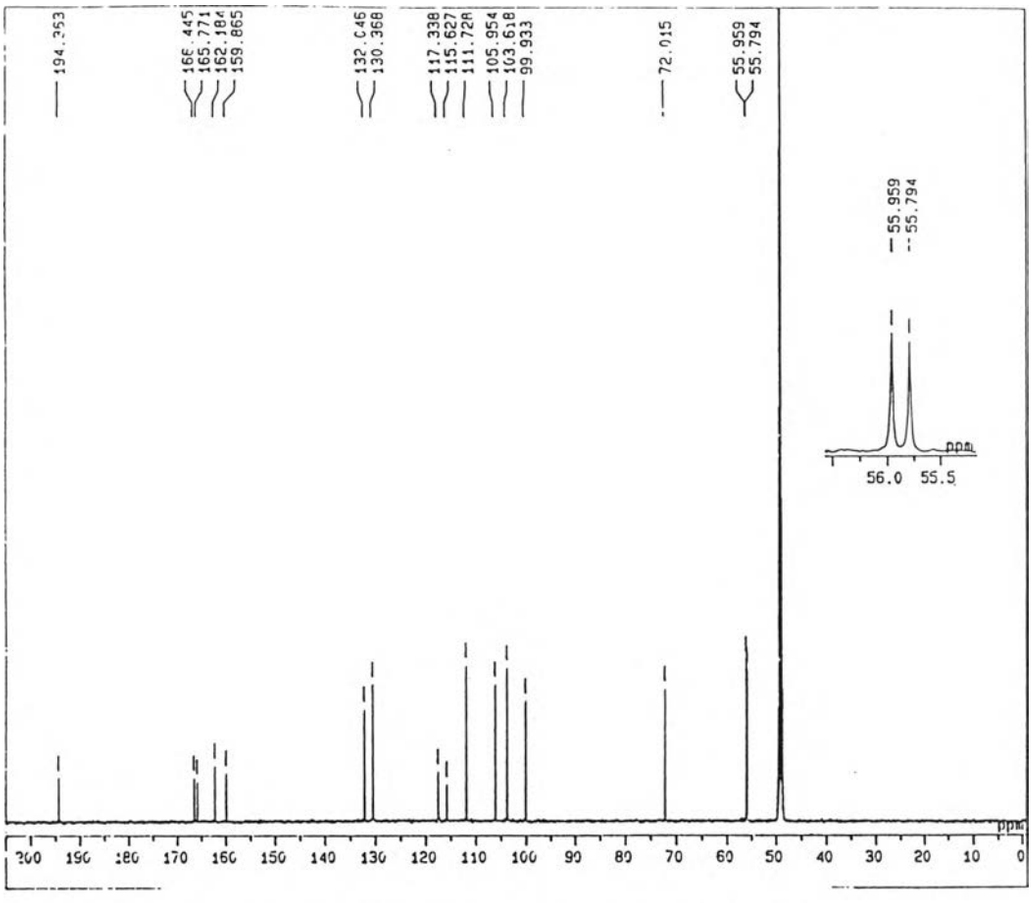


Figure 3.34 ^{13}C-NMR spectrum of compound 4

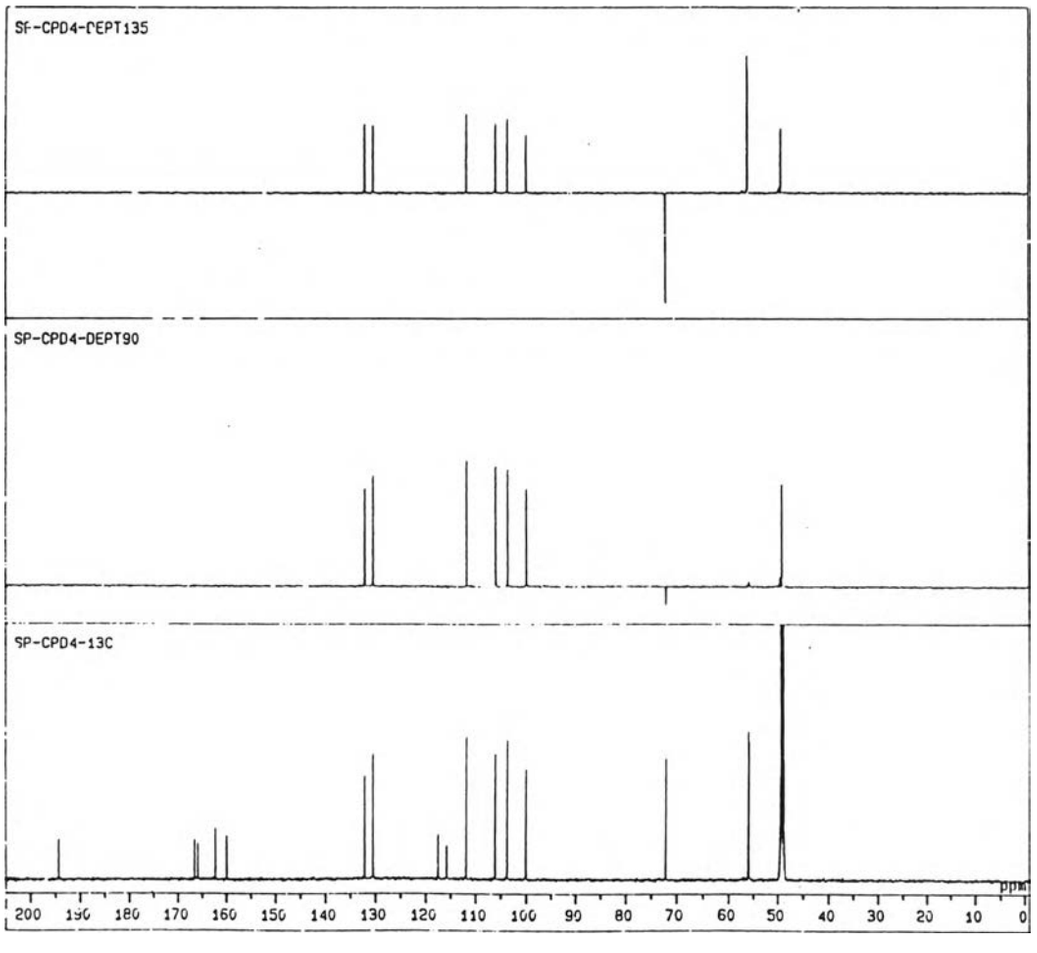


Figure 3.35 DEPT 90 and 135 spectrums of compound 4

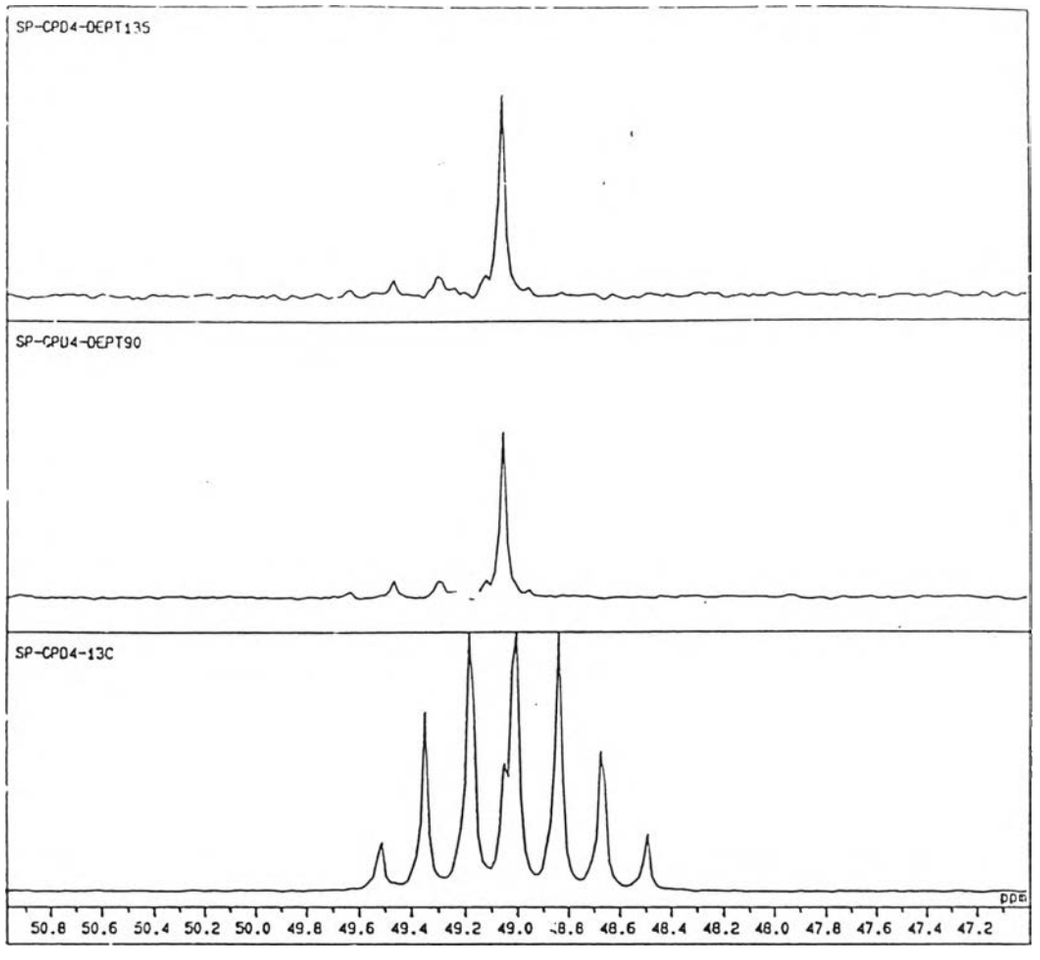


Figure 3.36 DEPT 90 and 135 spectrums of compound 4

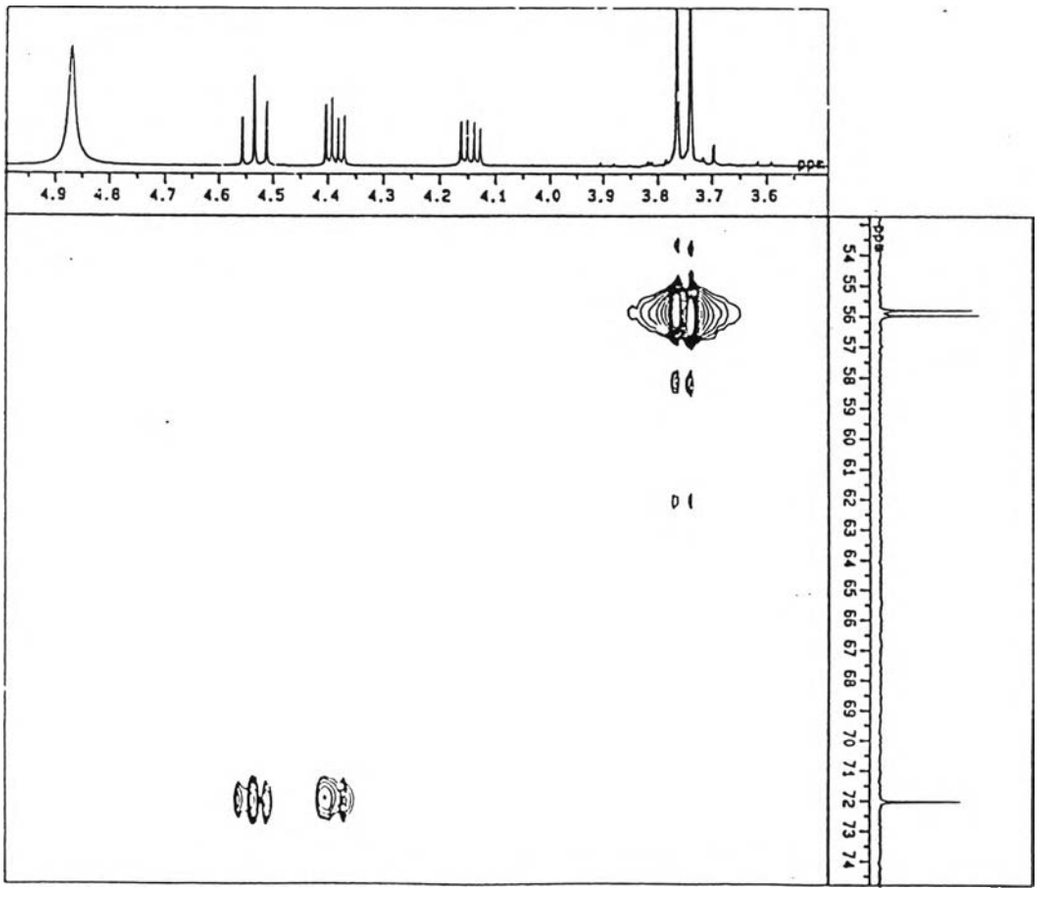


Figure 3.37 HMQC spectrum of compound 4

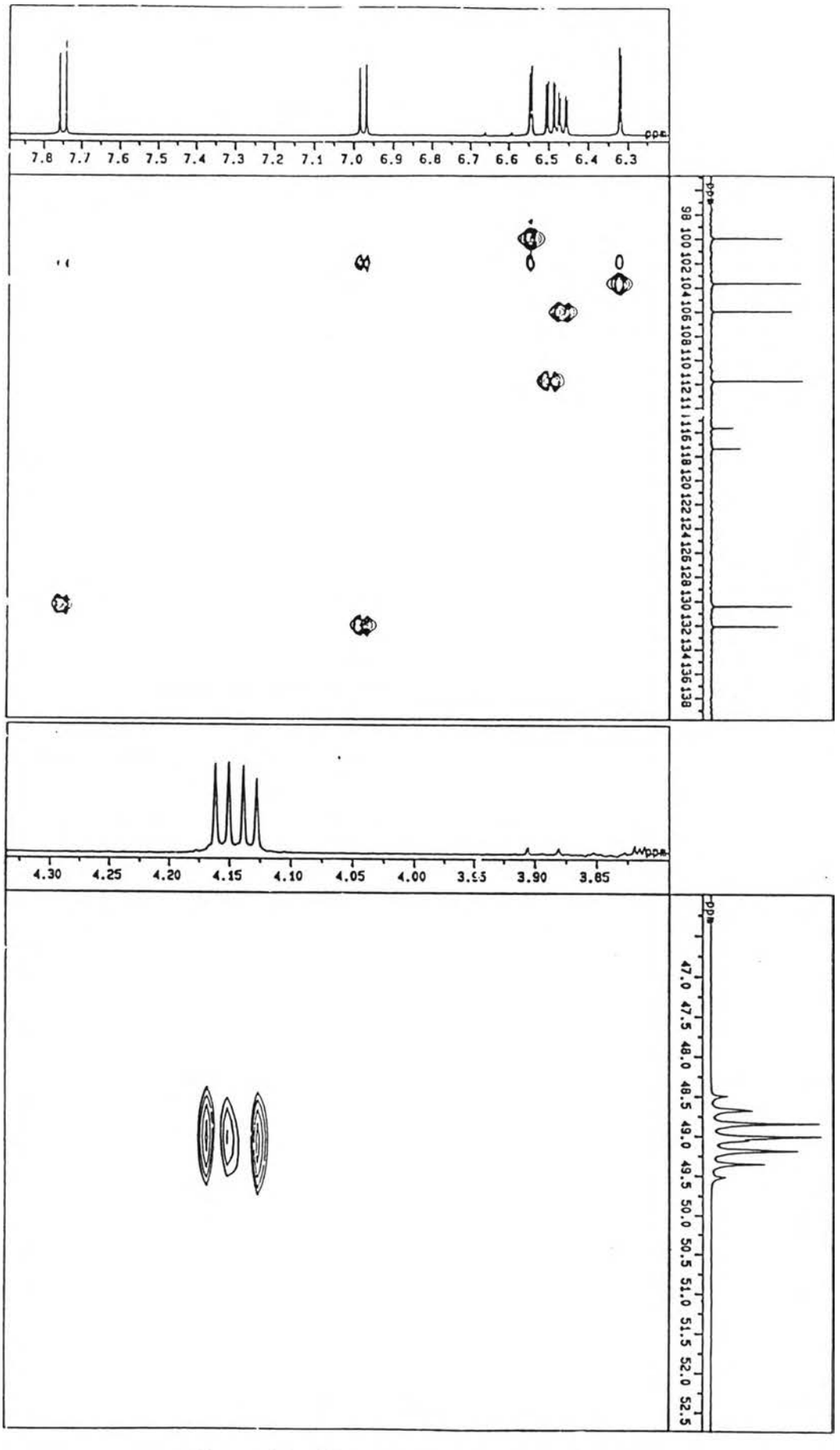


Figure 3.38 HMQC spectrum of compound 4

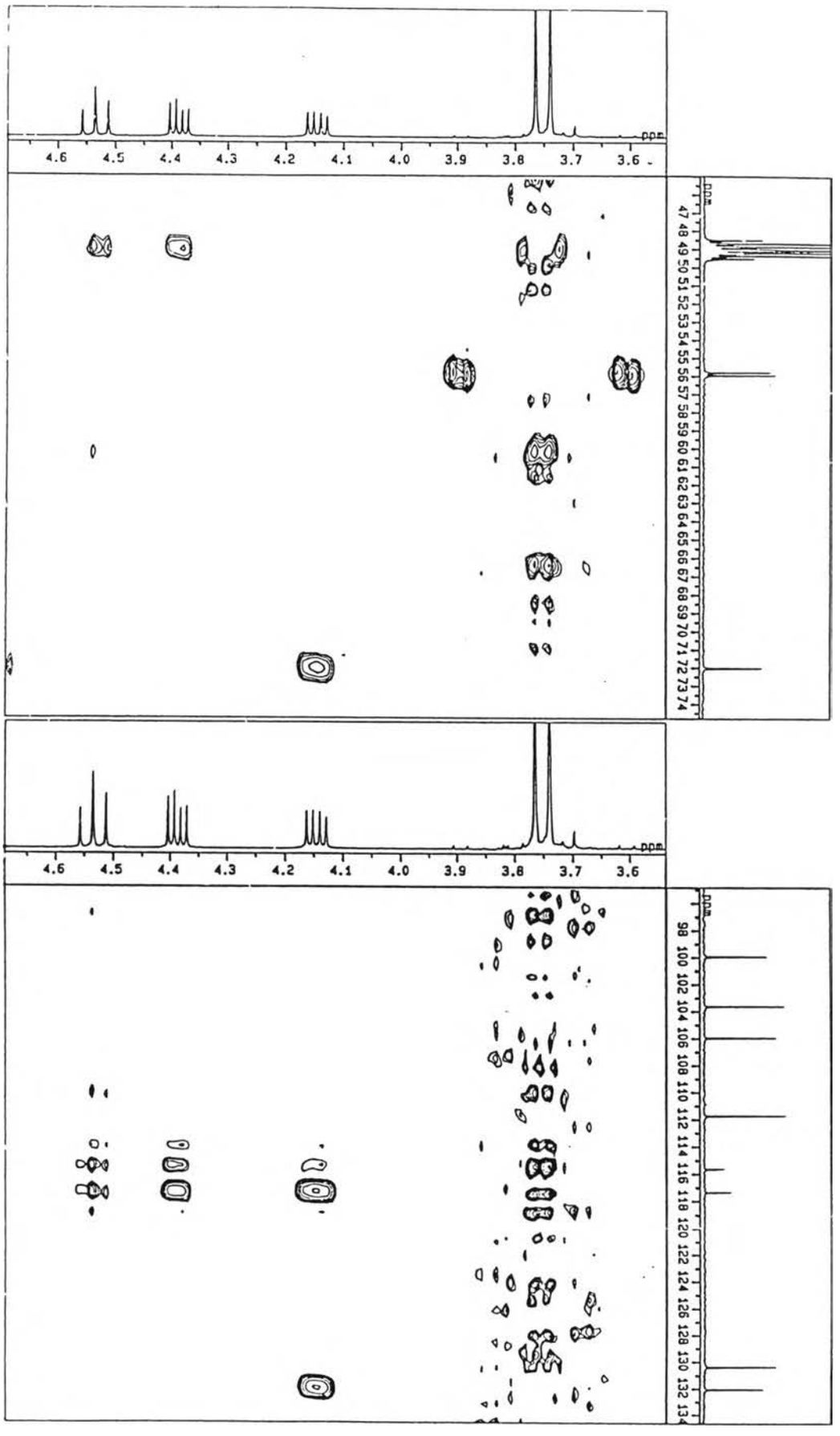


Figure 3.39 HMBC spectrum of compound 4

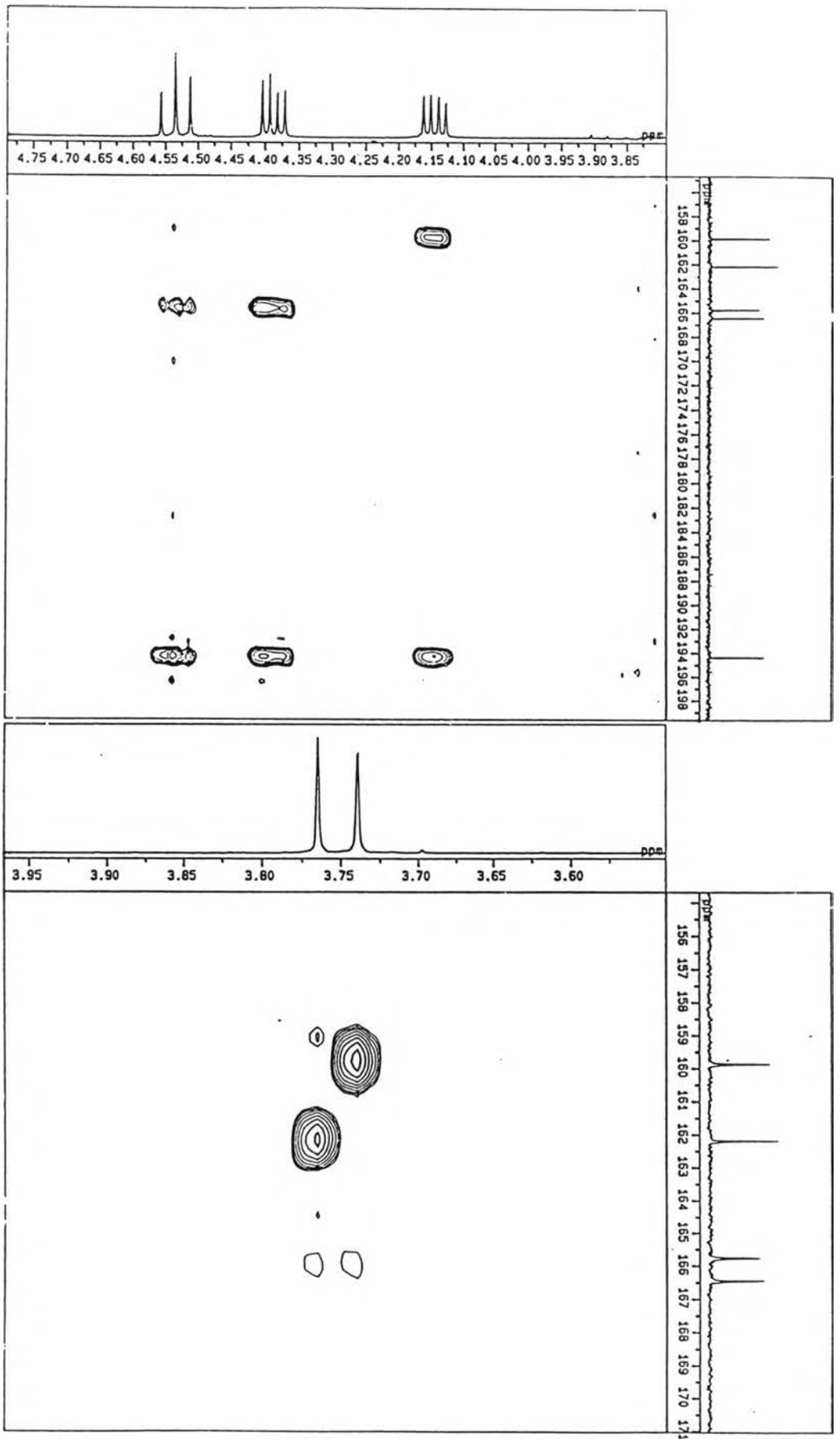


Figure 3.40 HMBC spectrum of compound 4

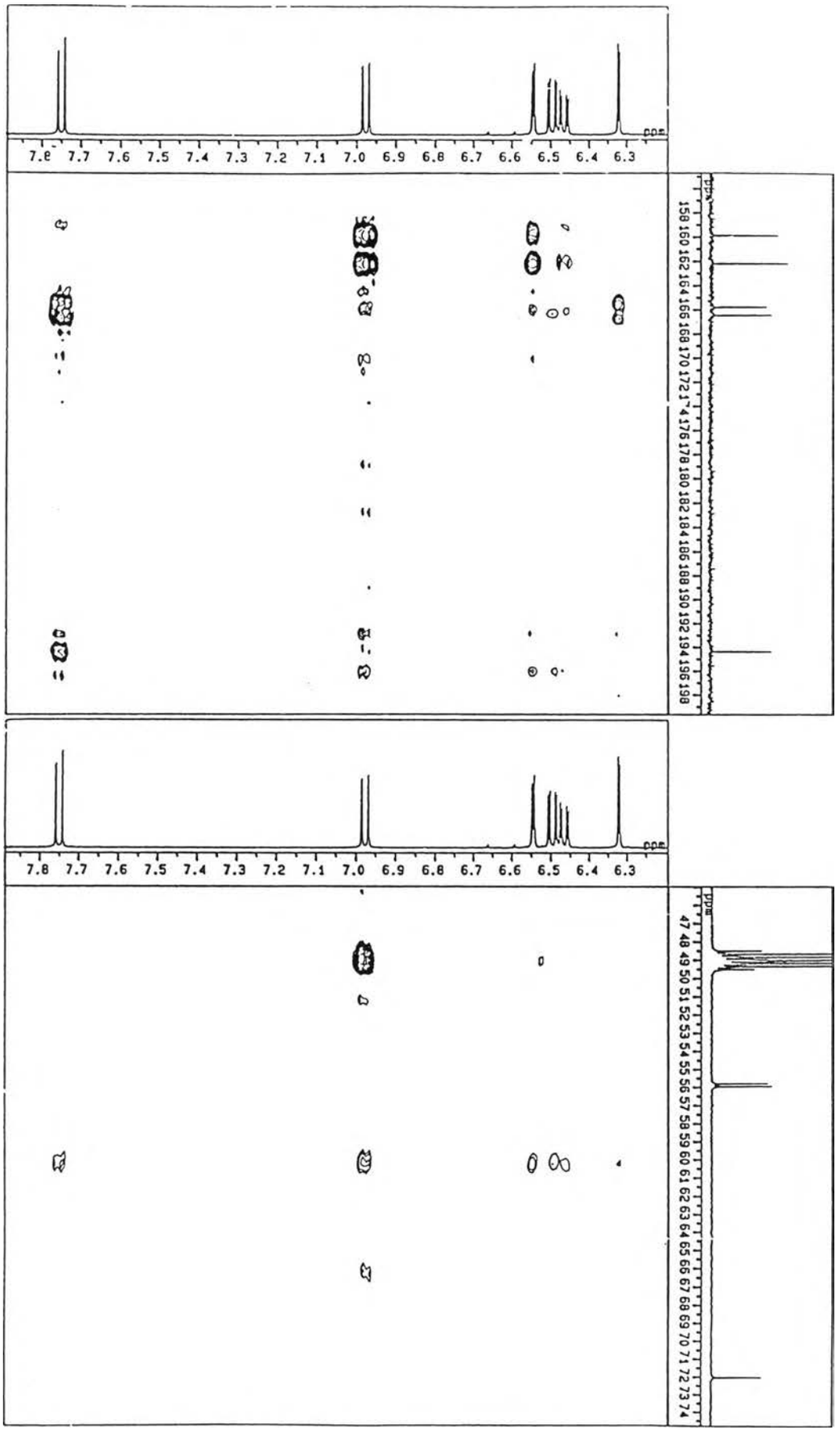


Figure 3.41 HMBC spectrum of compound 4

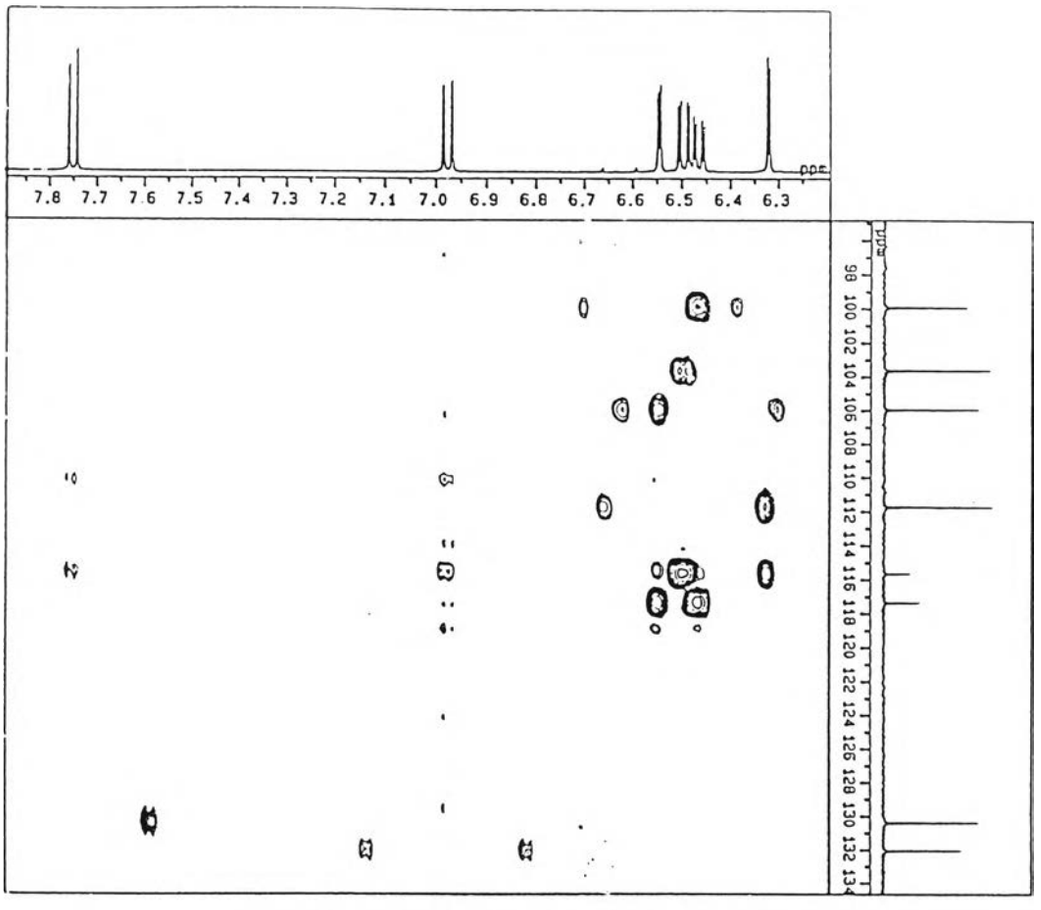


Figure 3.42 HMBC spectrum of compound 4

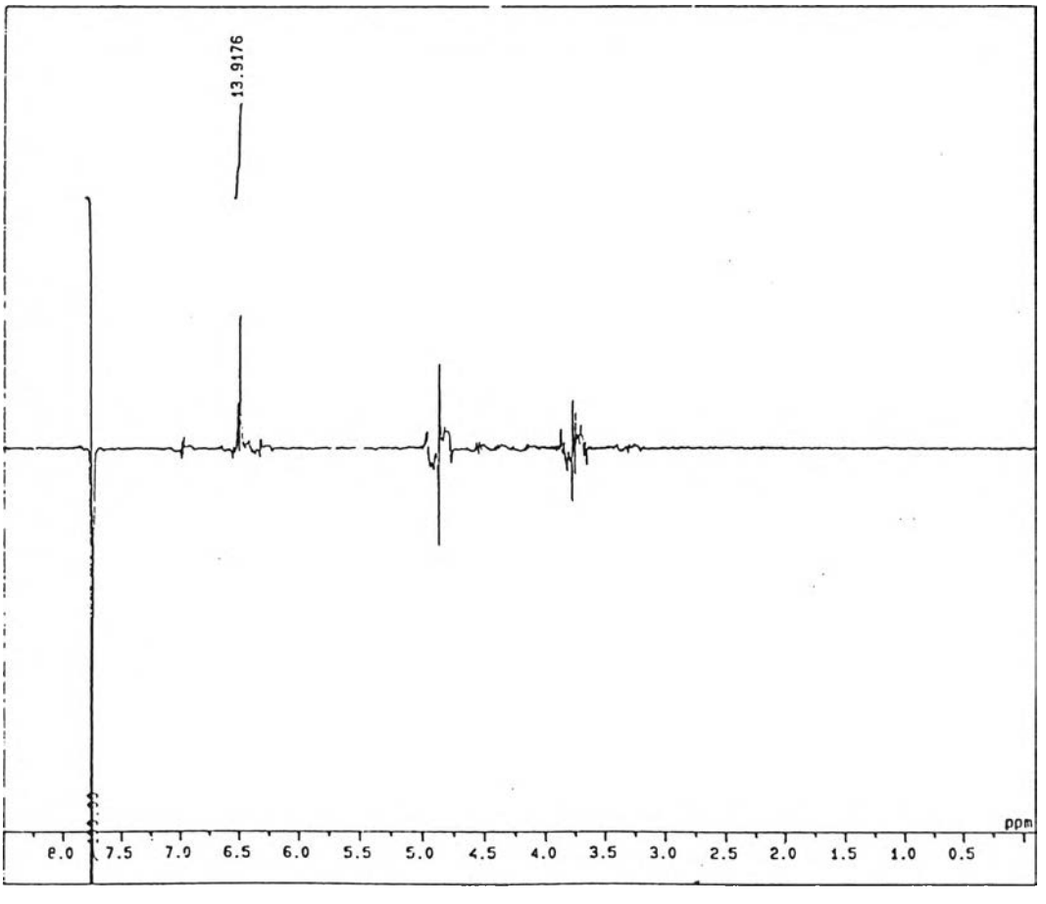


Figure 3.43 NOE DIFF of compound 4 (irradiated at 7.75 ppm)

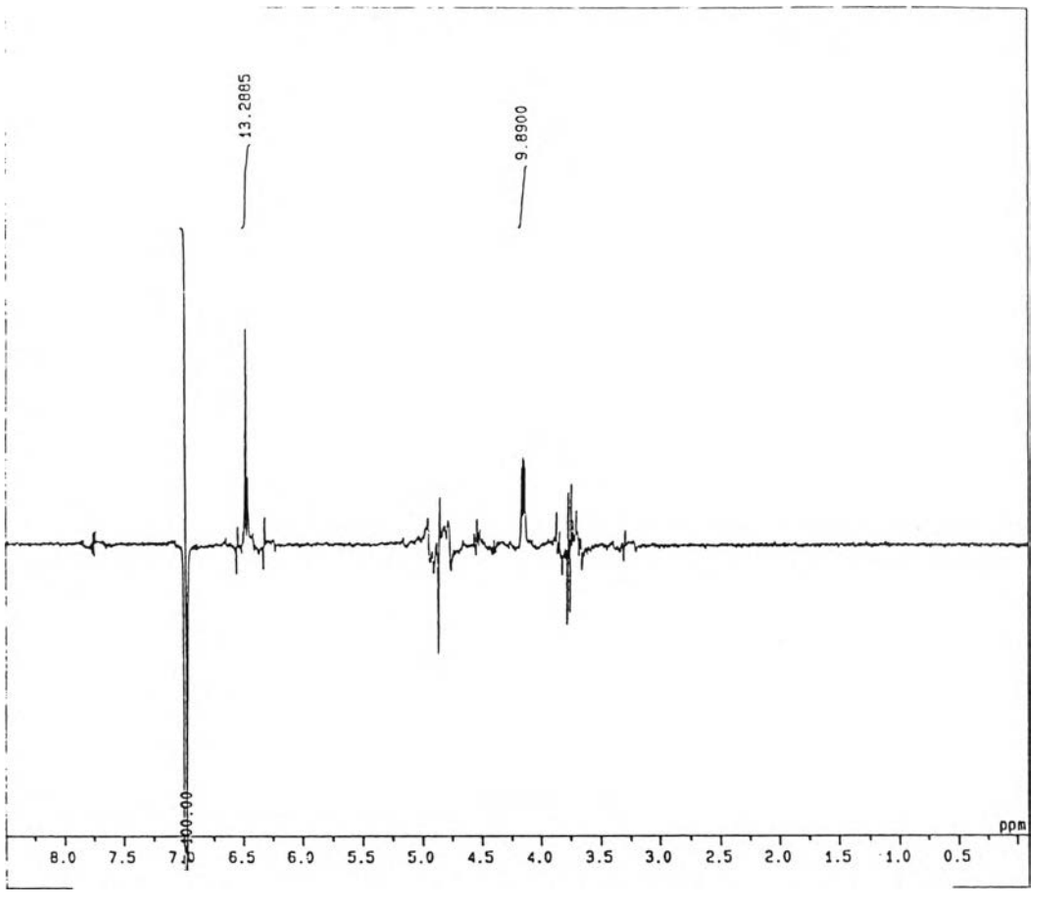


Figure 3.44 NOE DIFF of compound 4 (irradiated at 6.98 ppm)

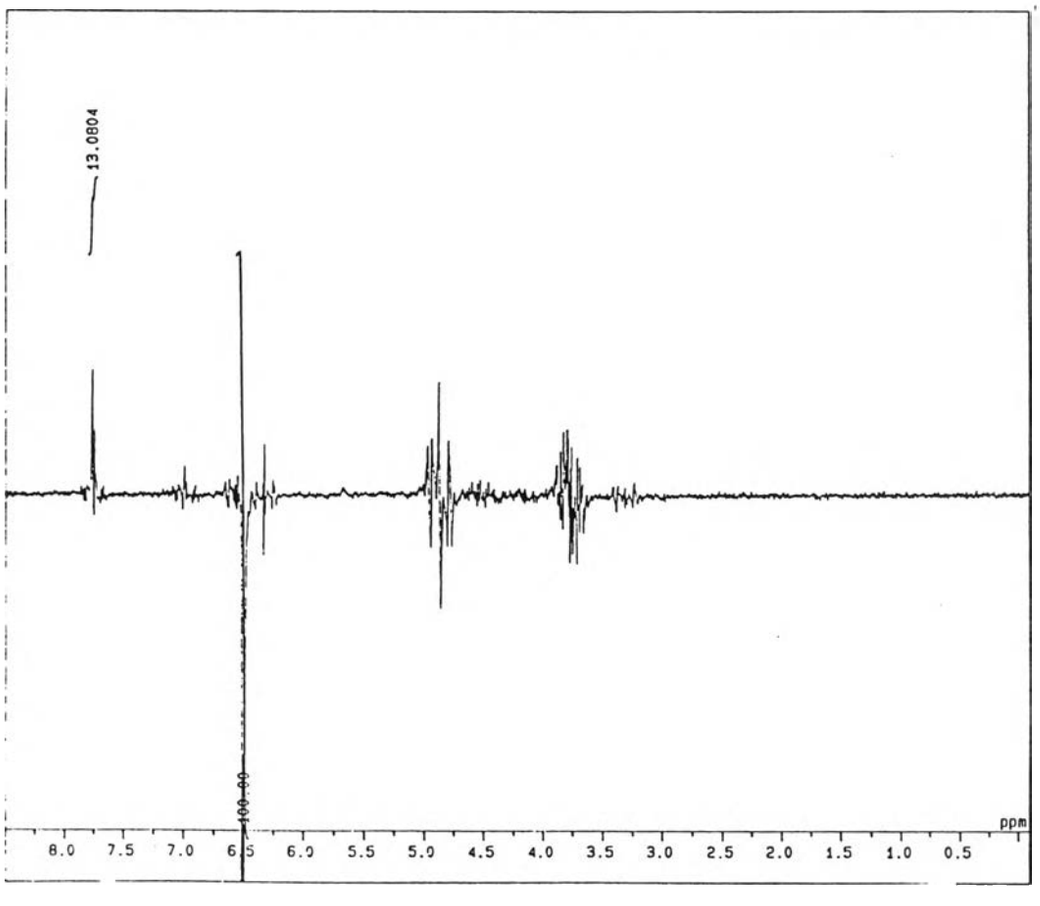


Figure 3.45 NOE DIFF of compound 4 (irradiated at 6.49 ppm)

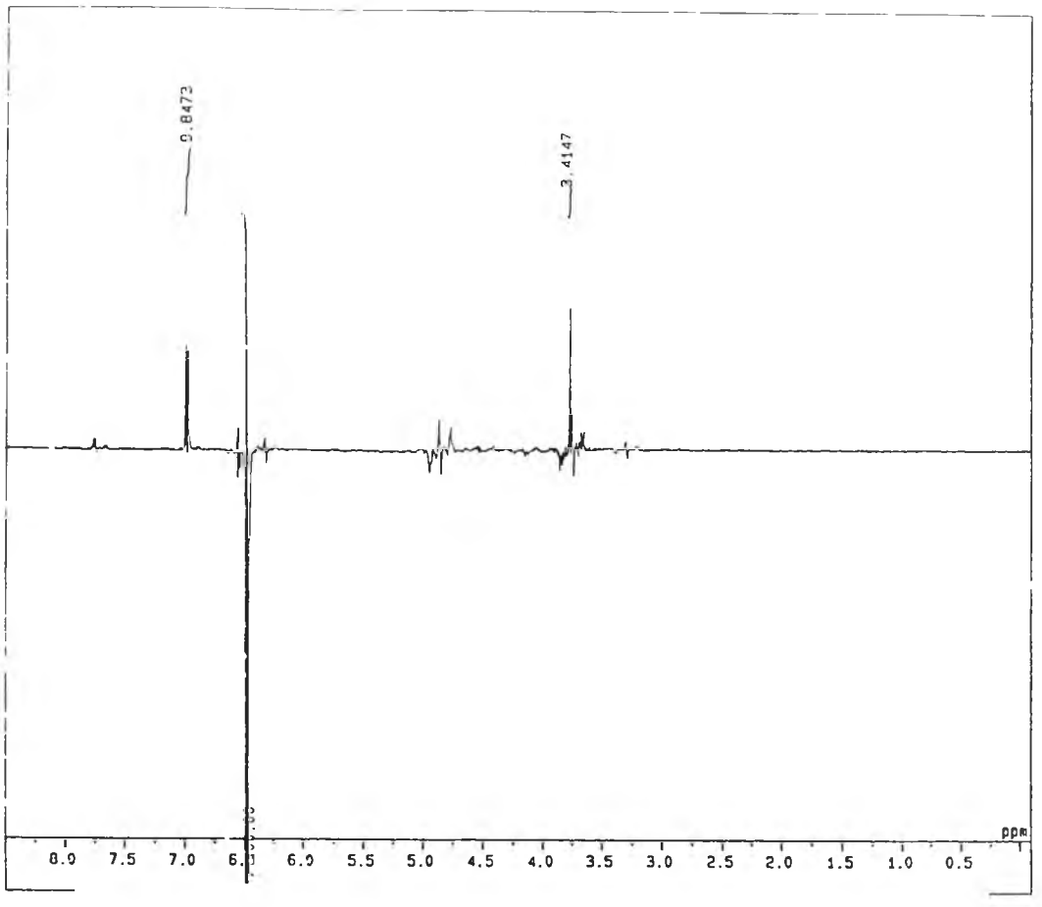


Figure 3.46 NOE DIFF of compound 4 (irradiated at 6.47 ppm)

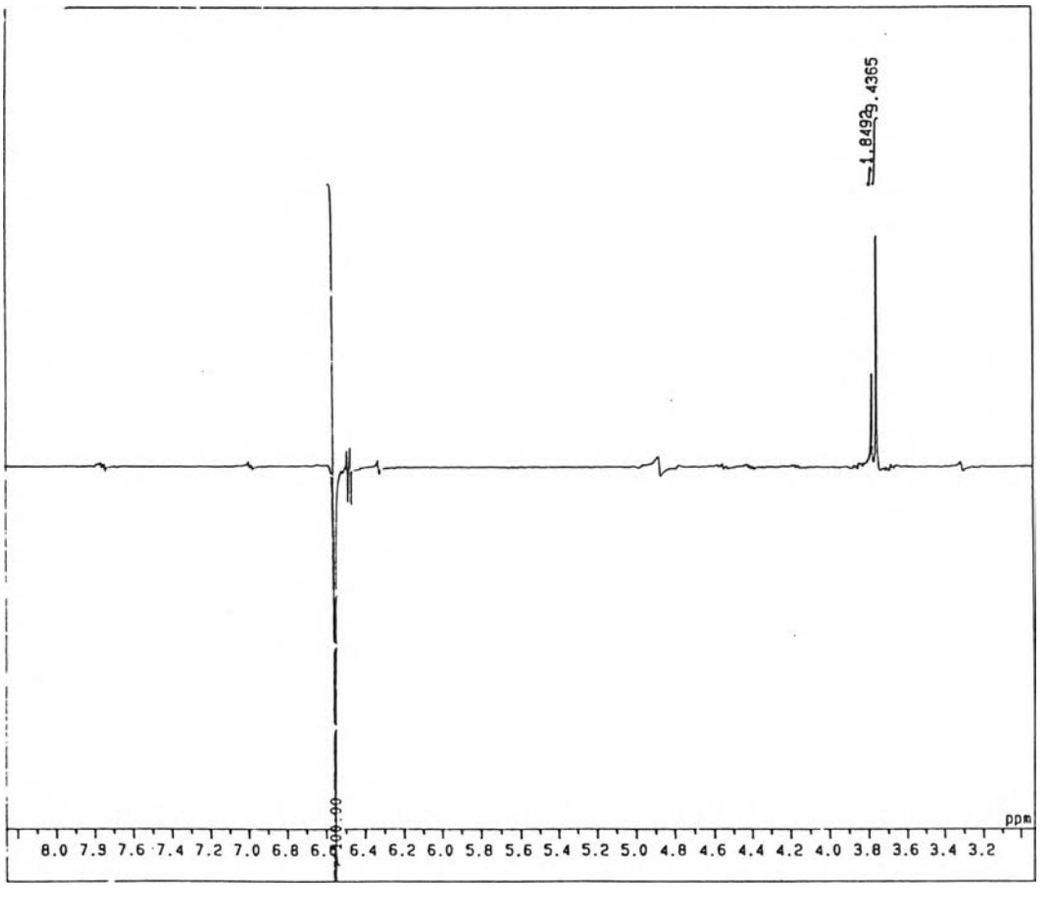


Figure 3.47 NOE DIFF of compound 4 (irradiated at 6.55 ppm)

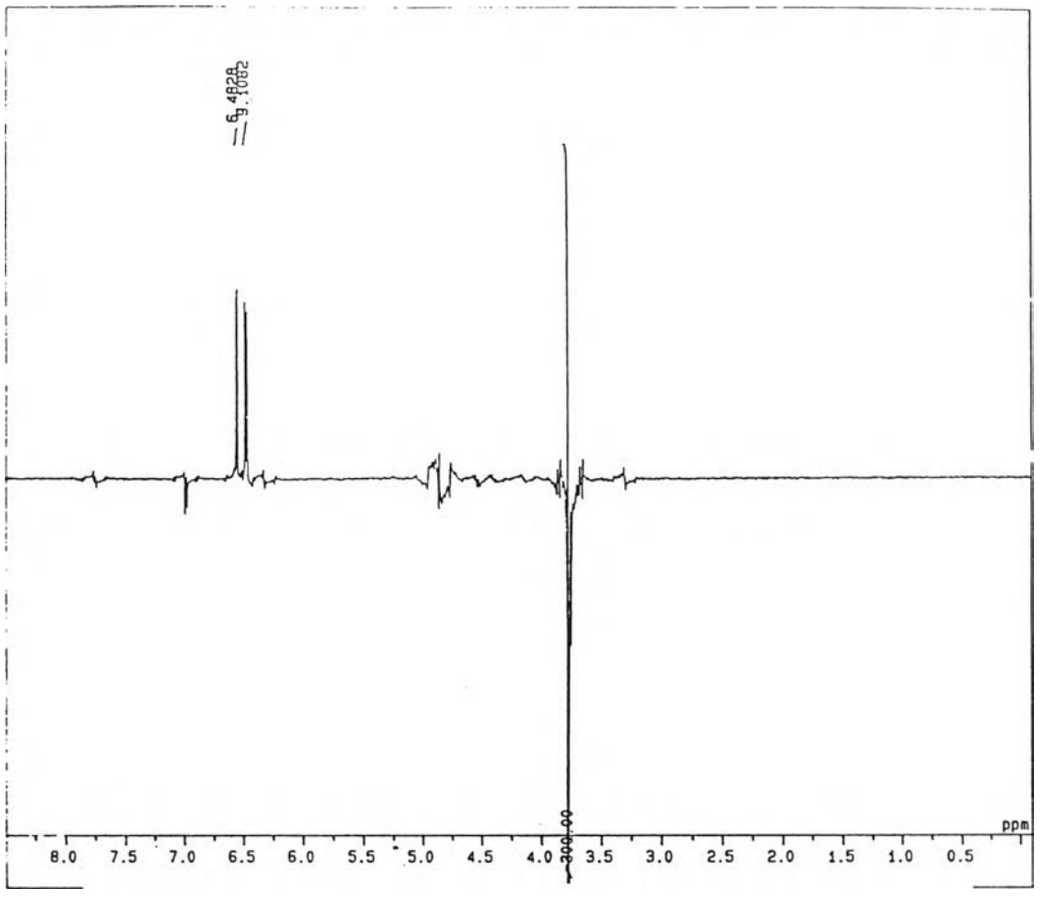


Figure 3.48 NOE DIFF of compound 4 (irradiated at 3.77 ppm)

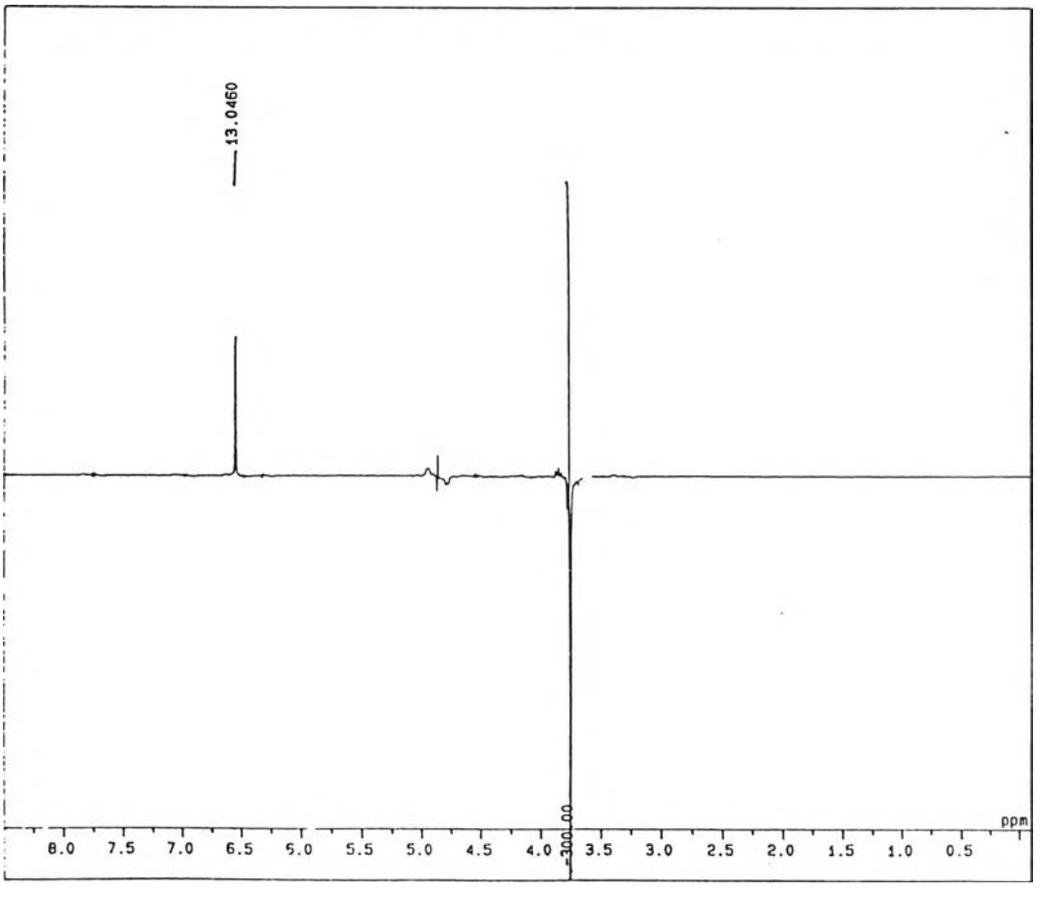


Figure 3.49 NOE DIFF of compound 4 (irradiated at 3.74 ppm)

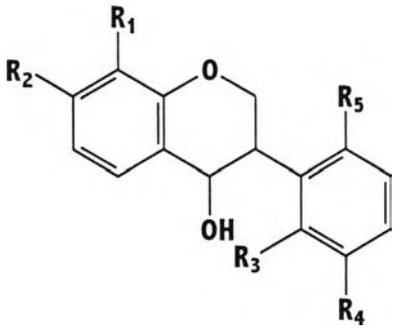
3.2.5 Compound 5

Compound 5 was isolated by open column chromatography of the crude dichloromethane extract and further purified by recrystalization (dichloromethane: methanol). This compound, m.p. 155-157 °C, was obtained as white needles, 67 mg. The Rf value was 0.46 (SiO_2, EtOAc-CH_2Cl_2, 1:9) and soluble in ethyl acetate and methanol, and slightly in dichloromethane. UV was λ_{max} 238 nm. The mass spectrum showed [M^+-H_2O] at m/z 316 (**Figure 3.52**).

The IR spectrum showed a board absorption band of hydroxy groups at 3600-3200 cm^{-1}, aromatic C=C bands at 1634, 1520, 1500 cm^{-1}, the C-O stretching band at 1096 cm^{-1} (**Figure 3.50**).

The ^1H NMR (CD_3OD) spectra contained four aromatic proton signals at δ 7.01 (1H, *d*, *J = 8.54 Hz*), 6.73 (1H, *d*, *J = 8.24 Hz*), 6.68 (1H, *d*, *J = 8.54 Hz*), 6.48 (1H, *d*, *J = 7.94 Hz*), suggestive of two ortho-aromatic protons in A and B ring, two methine protons at δ 5.50 (1H, *d*, *J = 6.72 Hz*) and 4.30 (1H, *m*)and one methylene proton at δ 3.50-3.58 (2H, *m*), typical of 4-hydroxyisoflavane and two methoxy protons at δ 3.83 (3H, *s*) and 3.79 (3H, *s*) (**Figure 3.53**).

The ^{13}C NMR spectrum (**Figure 3.51**) displayed seventeen carbon signals of fifteen carbons skeleton and two substitute groups (flavonoid characteristic); two methyl carbons of methoxy groups at δ 56.9 (OCH_3) and 56.6 (OCH_3) ppm. The analysis of this spectrum by the aid of DEPT 90, 135 (**Figure 3.54**) technique unequivocally indicated that compound 5 contained eight quarternary carbons at δ 115.4 (C-10), 122.6 (C-1'), 132.4 (C-5'), 135.6 (C-8), 145.4 (C-9), 148.2 (C-6'), 149.5 (C-7), 150.4 (C-1') ppm, six methine carbons at δ 41.5 (C-3), 80.3 (C-4), 105.8 (C-4'), 106.7 (C-6), 115.6 (C-5), 122.2 (C-3') ppm, one methylene carbon at δ 67.8 (C-2) ppm. These results suggested that compound 5 was $C_{17}H_{18}O_7$ on the basis of ^1H, ^{13}C NMR and EIMS, confirmed by the elemental analysis (C 61.27 %, H 5.41 %, O 33.32 %, calculated for C 61.07 %, H 5.43 %, O 33.50 %). Therefore, all of the above data could suggest, the possibility of a flavonoid structure of isoflavanol with two substituted A ring and three substituted B ring, the molecule of this compound must have the basic skeleton showed below:

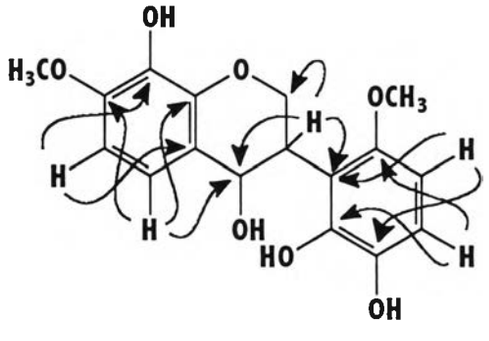


Substituents;

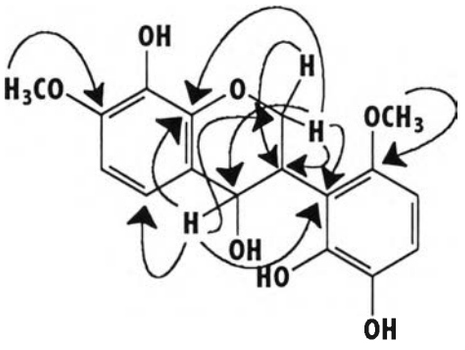
-OH (3)

-OCH_3 (2)

In the H-C long range coupling spectrum obtained by HMBC (Heteronuclear multiple bond correlation), the aromatic proton at δ 6.68 ppm (H-6) showed cross peaks with the carbons at δ 135.6 (C-8), 115.4 (C-10), the proton at δ 7.01 ppm showed cross peaks with the carbons at δ 149.5 (C-7), 145.4 (C-9) and 80.3 (C-4). The proton at δ 6.73 ppm showed cross peaks with the carbons at δ 148.2 (C-6') and 150.4 (C-2') ppm, the proton at δ 6.48 ppm showed cross peaks with the carbons at δ 122.6 (C-1') and 132.4 (C-5'), the proton at δ 5.50 ppm showed cross peaks with the carbons at δ 67.8 (C-2), 115.6 (C-5), 145.4 (C-9) and 122.6 (C-1') ppm, the proton at δ 3.52 ppm showed cross peaks with carbons at δ 67.8 (C-2), 122.6 (C-1') and 80.3 (C-4). The methylene protons were unequal H_a at δ 3.56 ppm showed cross peak with carbon at δ 41.5 (C-3) and H_b at δ 4.30 ppm showed cross peaks with carbons at δ 41.5 (C-3), 80.3 (C-4), 145.4 (C-9) and 122.6 (C-1'), the protons in methoxy group at δ 3.79 ppm showed cross peak with carbon at δ 150.4 (C-2') ppm and the protons in methoxy group at δ 3.83 ppm showed cross peak at δ 149.5 (C-7) ppm (**Figure 3.55-3.59**).



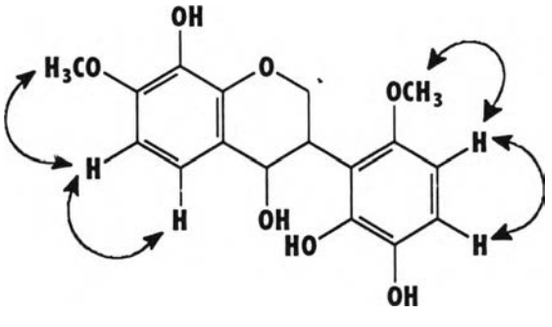
The HMBC correlation of compound 5



The HMBC correlation of compound 5

NOE DIFF experiments were used to obtain additional information. Irradiation of the proton at δ 7.01 ppm caused enhancement of the signal at δ 5.50 and 6.68 ppm while irradiation of the proton at δ 6.73 ppm caused enhancement of the signal at δ 6.48 ppm. When the protons of two methoxy groups at δ 3.83 and 3.79 ppm were irradiated, the enhancements of the signal at δ 6.68 (H-6), 6.48 (H-3') ppm were also observed, respectively (**Figure 3.60-3.65).**

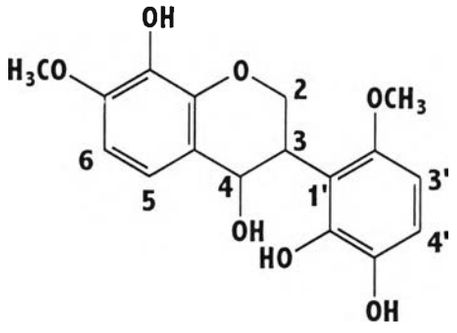
The NOE DIFF data indicated that two methoxy groups were next to the protons at δ 6.48 (H-3') and 6.68 (H-6) ppm



The NOE DIFF of compound 5

Confirmation of the A and B rings substitution patterns were accomplished from the long-range heteronuclear coupling observed in the HMBC spectrum as well as with NOE DIFF experiment.

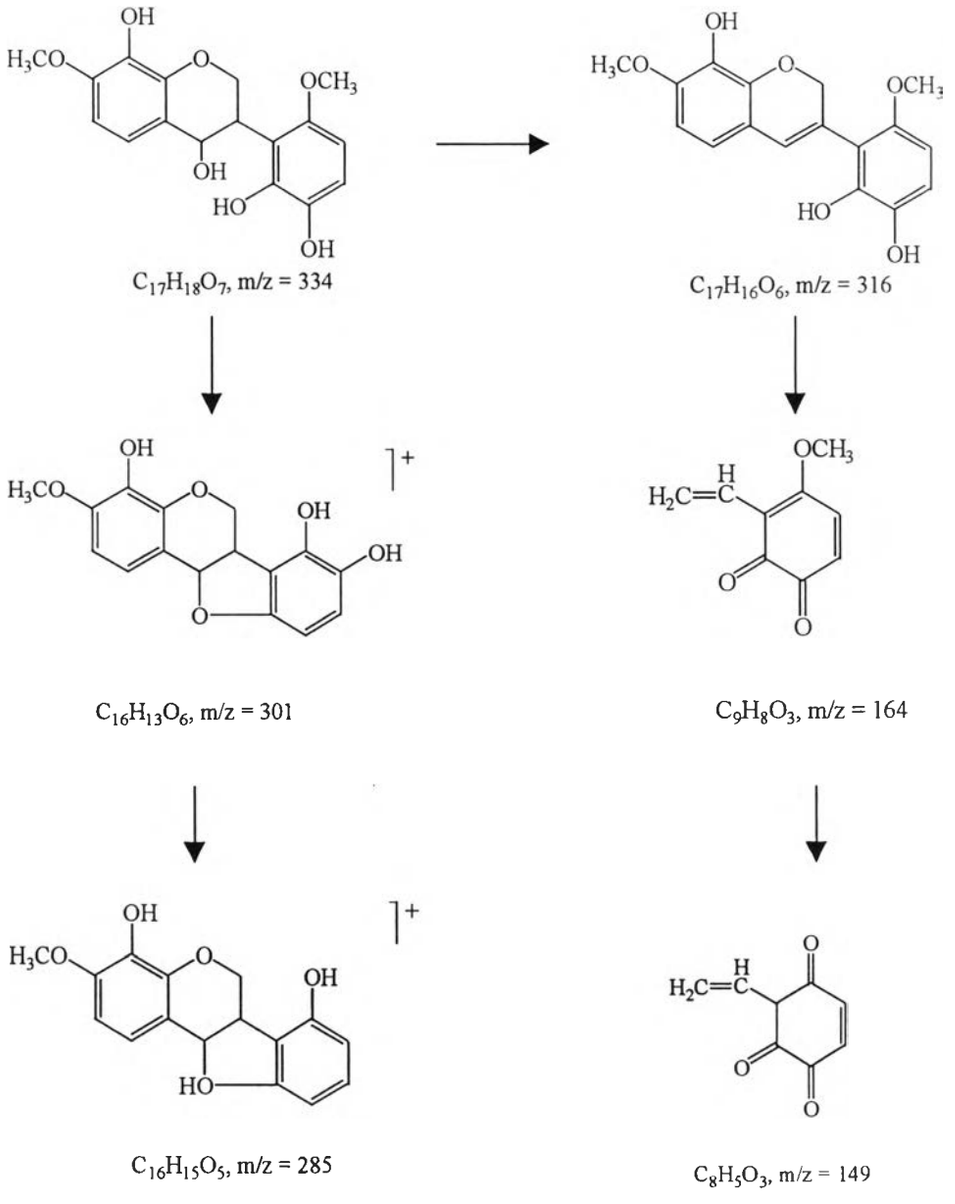
All of the spectral data was consistent with the identification of compound 5 as 8,5',6'-trihydroxy-7,2'-dimethoxyisoflavan-4-ol. From our knowledge, this compound was a new compound.



The structure of compound 5

Table 3.9 ^{1}H, ^{13}C and HMBC spectral data of compound 5 in CD_3OD (500 MHz)

C	δ (ppm)		HMBC (H → C)
	^{13}C	^{1}H	
2	67.8	3.56	C-3
		4.30	C-3, C-4, C-9, C-1'
3	41.5	3.52	C-2, C-4, C-1'
4	80.3	5.50	C-2, C-5, C-9, C-1'
5	115.6	7.01	C-4, C-7, C-9
6	106.7	6.68	C-8, C-10
7	149.5	-	-
8	135.6	-	-
9	145.4	-	-
10	115.4	-	-
1'	122.6	-	-
2'	150.4	-	-
3'	122.2	6.48	C-1', C-5'
4'	105.8	6.73	C-2', C-6'
5'	132.4	-	-
6'	148.2	-	-
$-OCH_3$	56.9	3.83	C-7
$-OCH_3$	56.6	3.79	C-2'



Scheme 7 Possible mass fragmentation patterns of compound 5

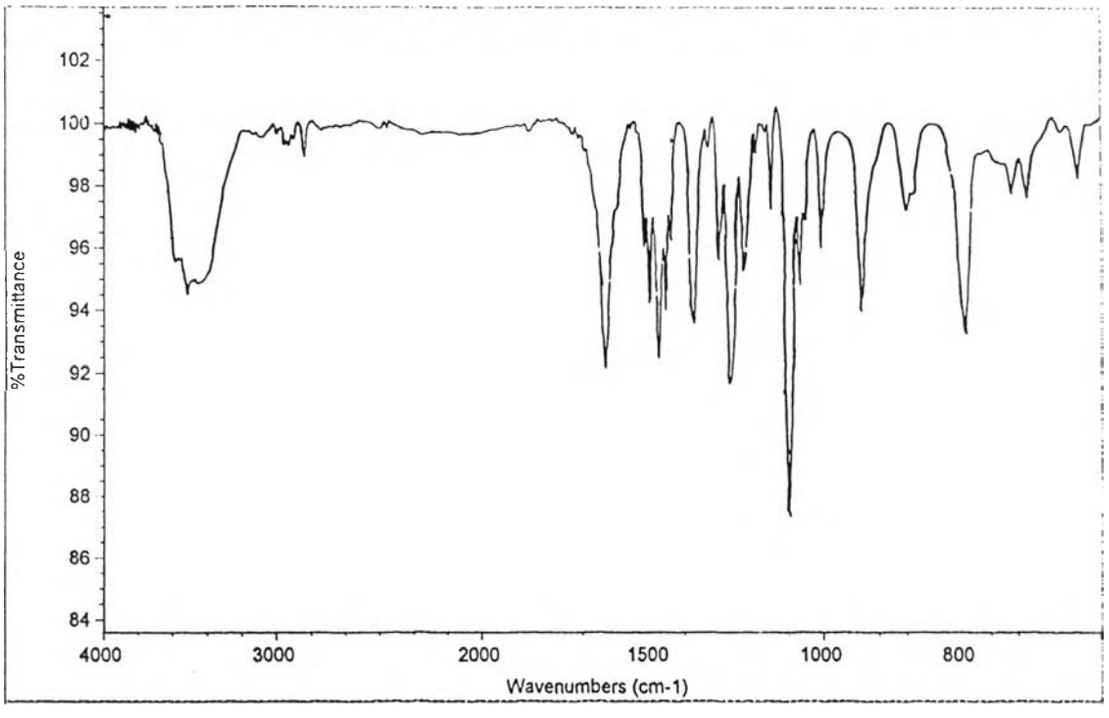


Figure 3.50 IR spectrum of compound 5

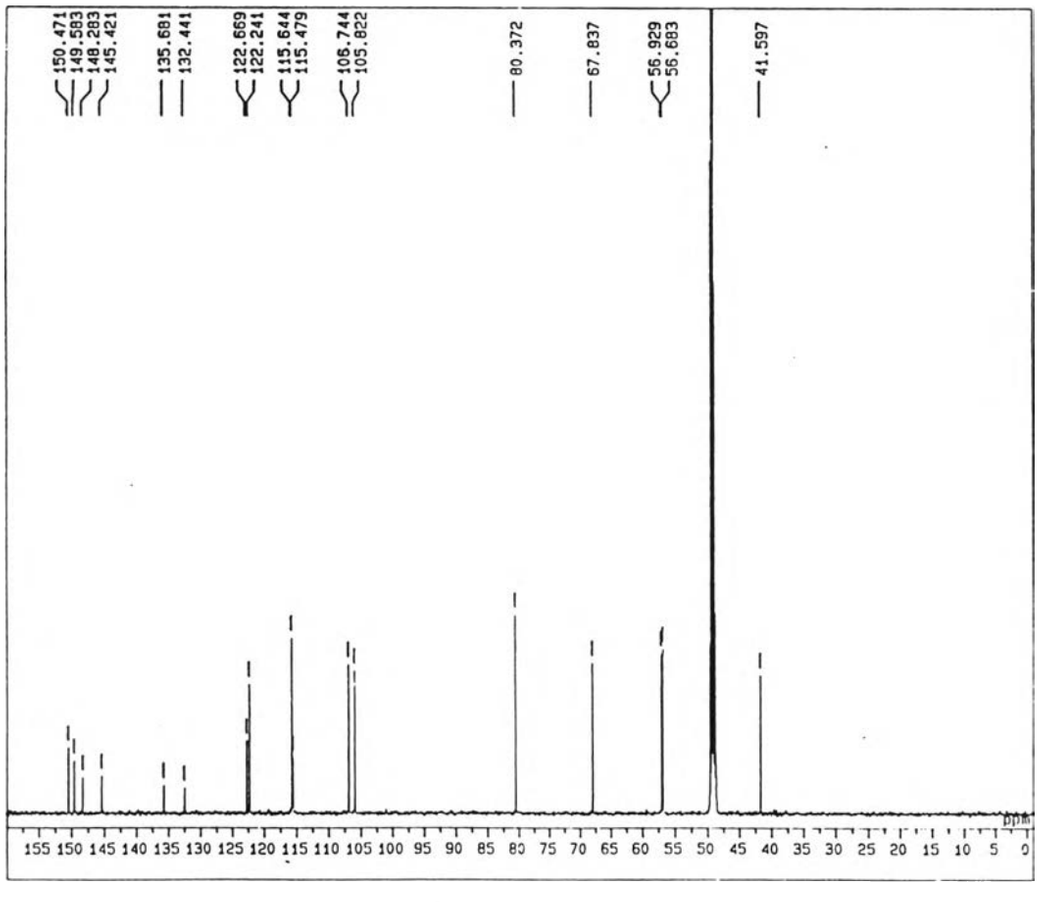


Figure 3.51 ^{13}C-NMR spectrum of compound 5

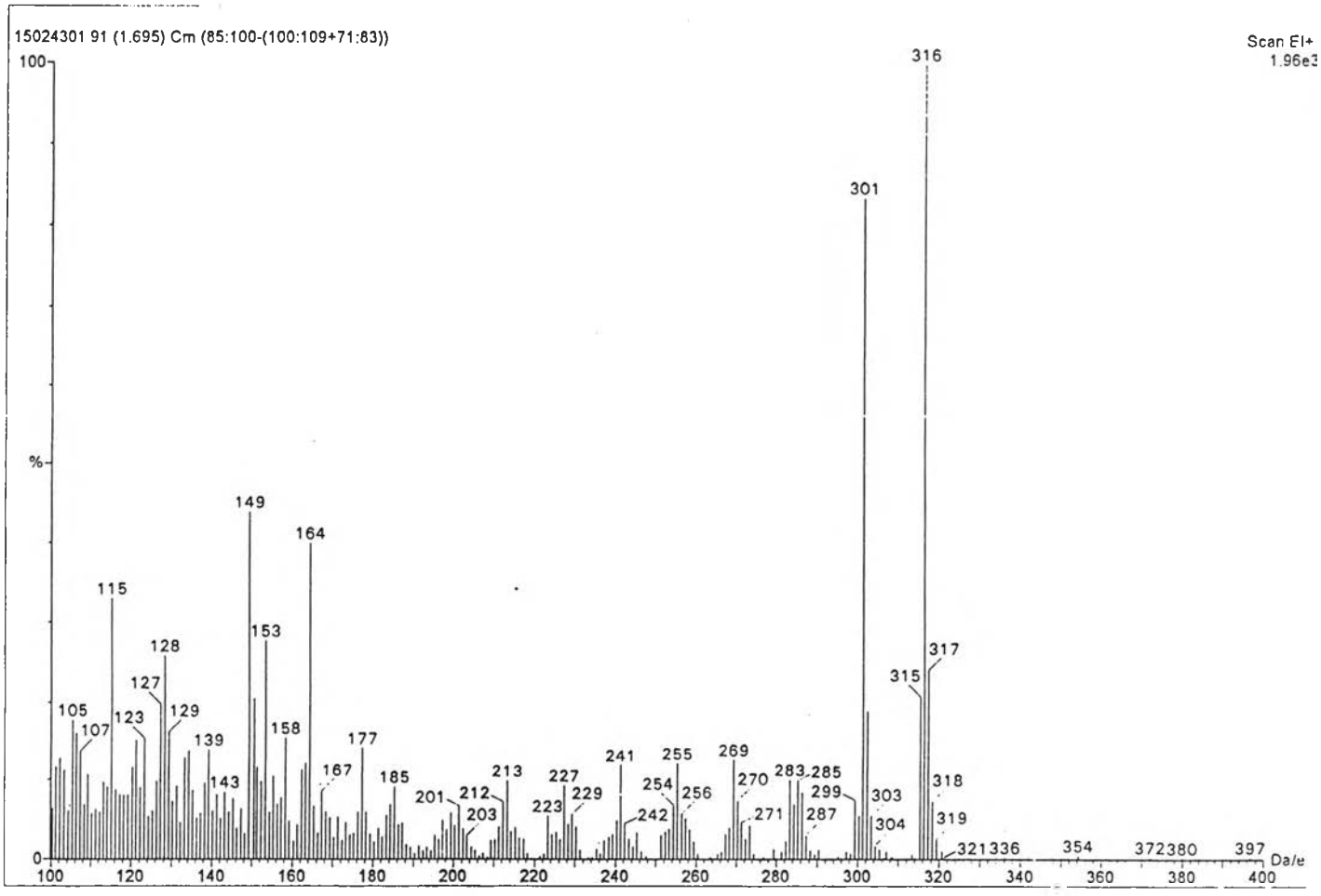


Figure 3.52 Mass spectrum of compound 5

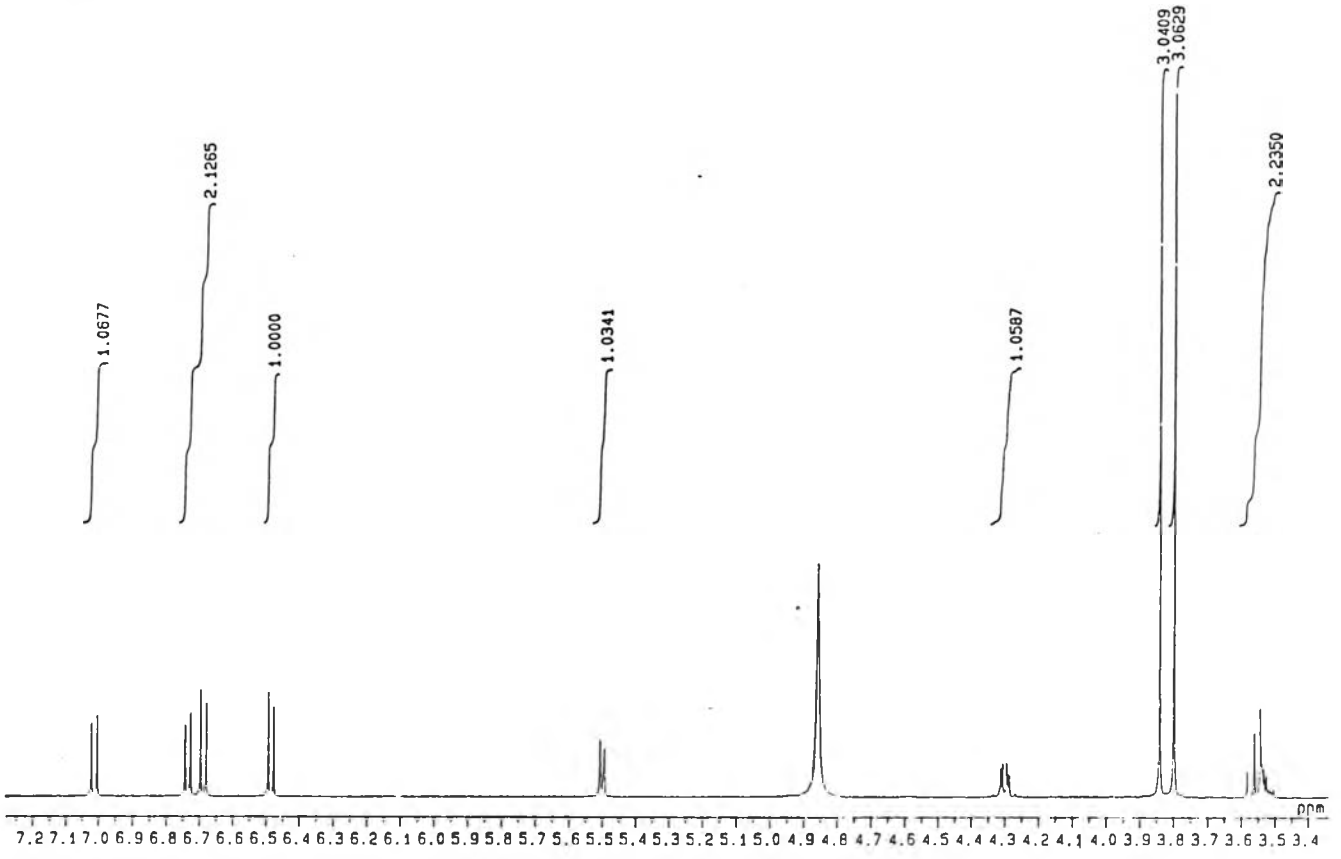


Figure 3.53 ^{1}H-NMR spectrum of compound 5

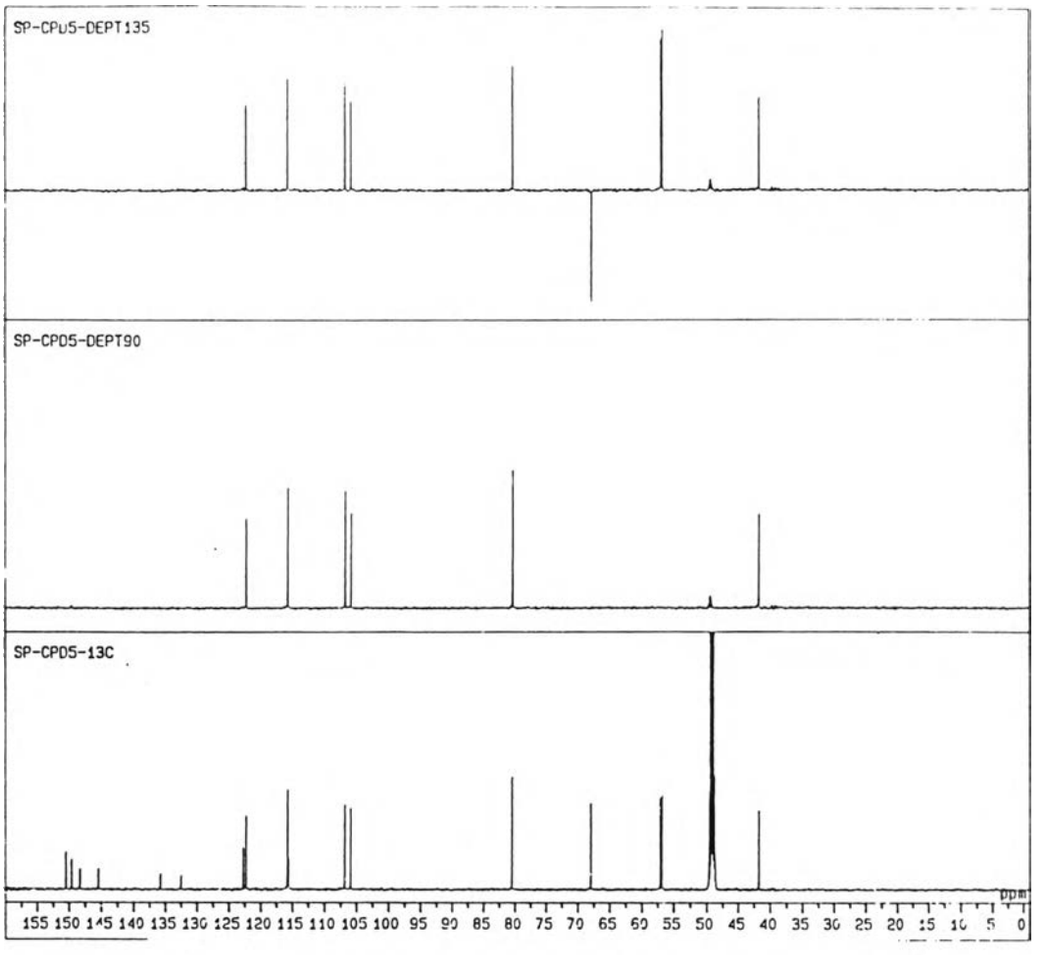


Figure 3.54 DEPT 90 and 135 spectrums of compound 5

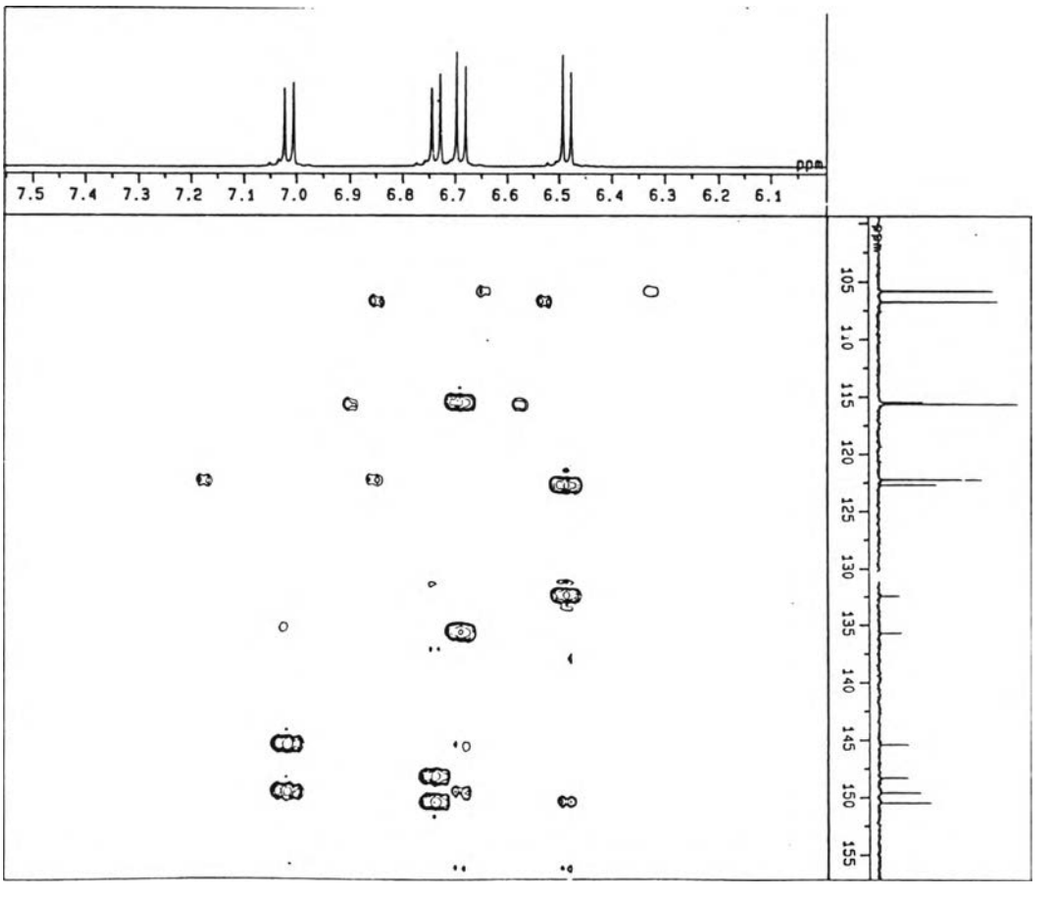


Figure 3.55 HMBC spectrum of compound 5

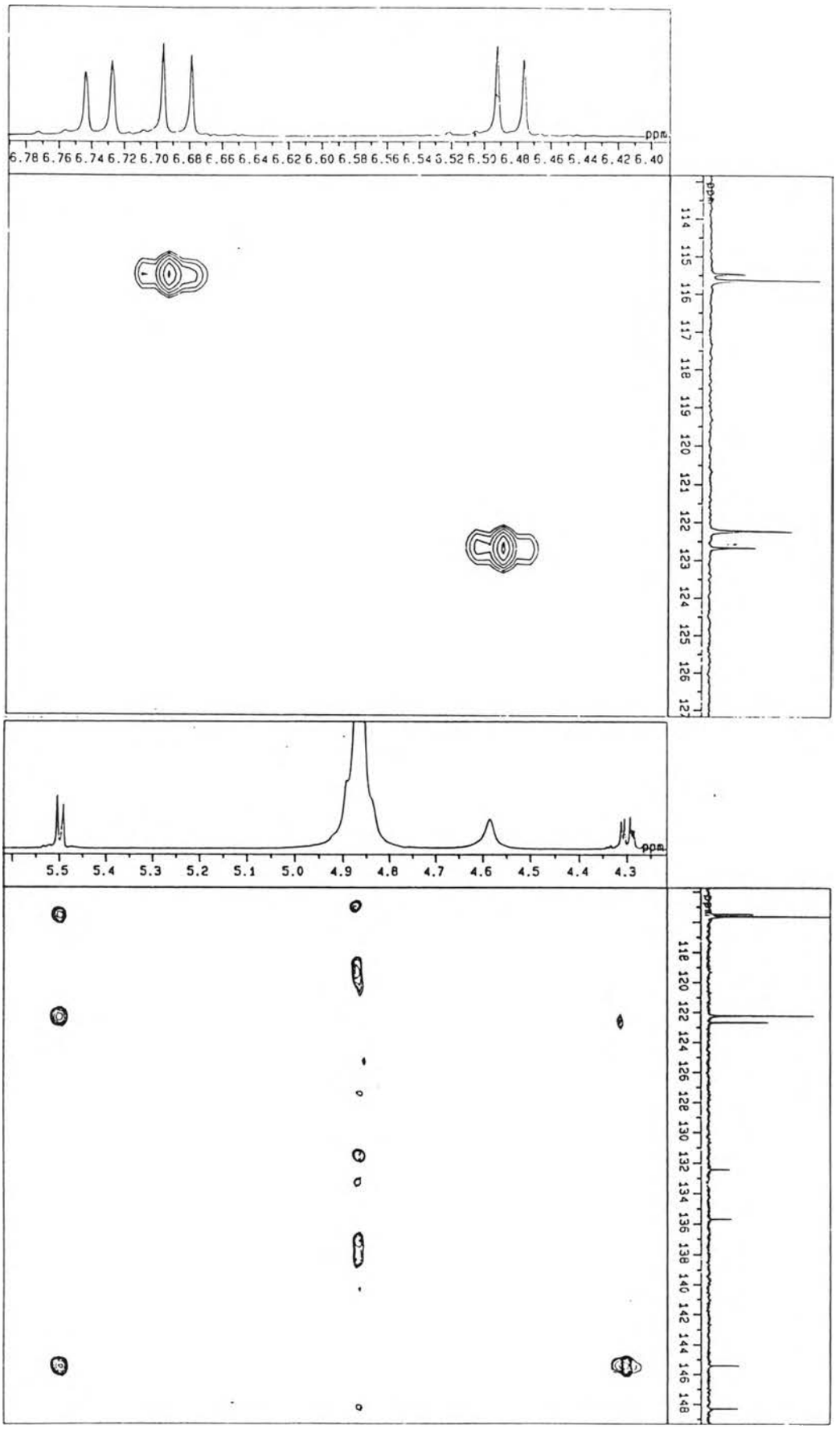


Figure 3.56 HMBC spectrum of compound 5

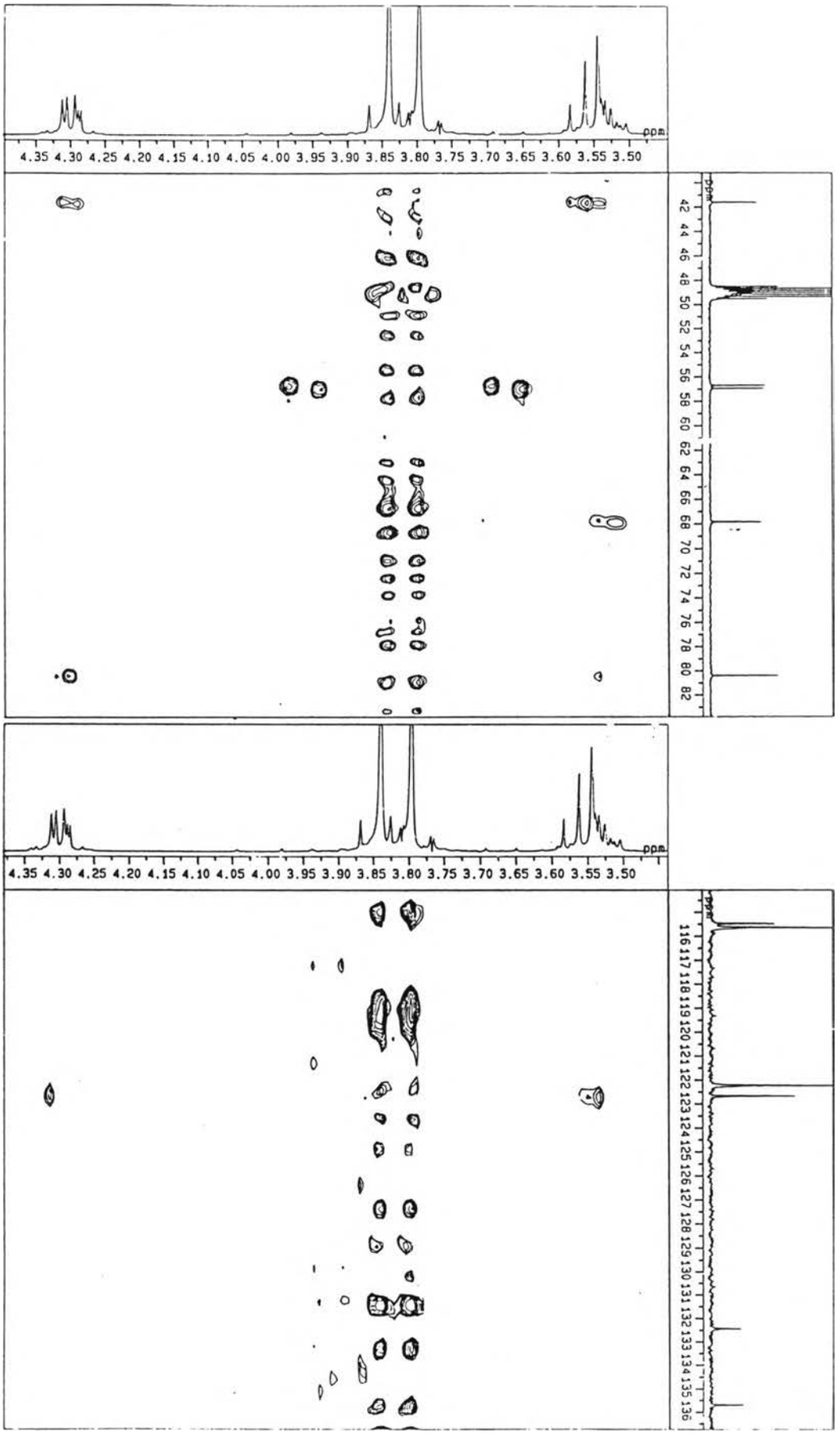


Figure 3.57 HMBC spectrum of compound 5

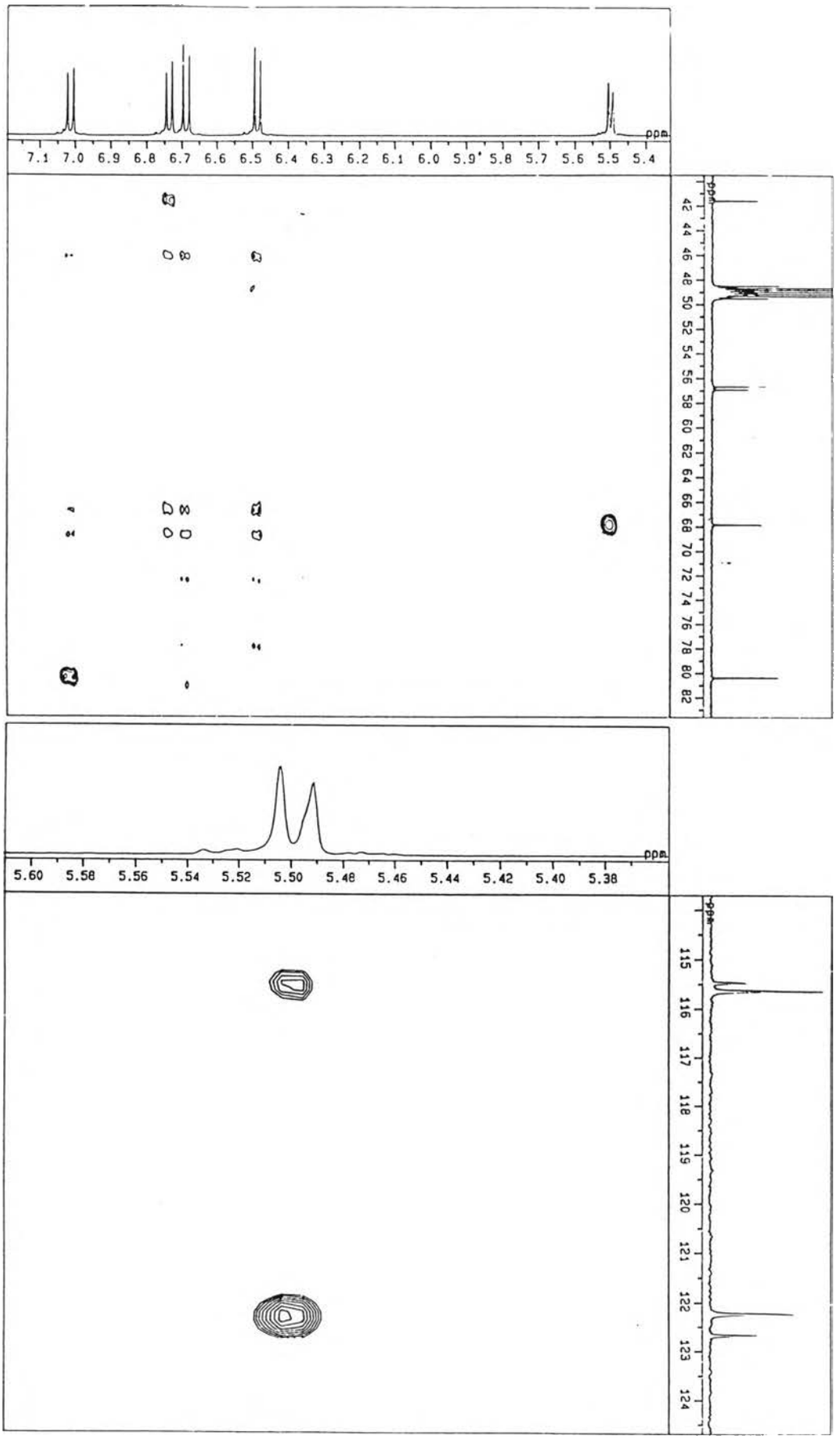


Figure 3.58 HMBC spectrum of compound 5

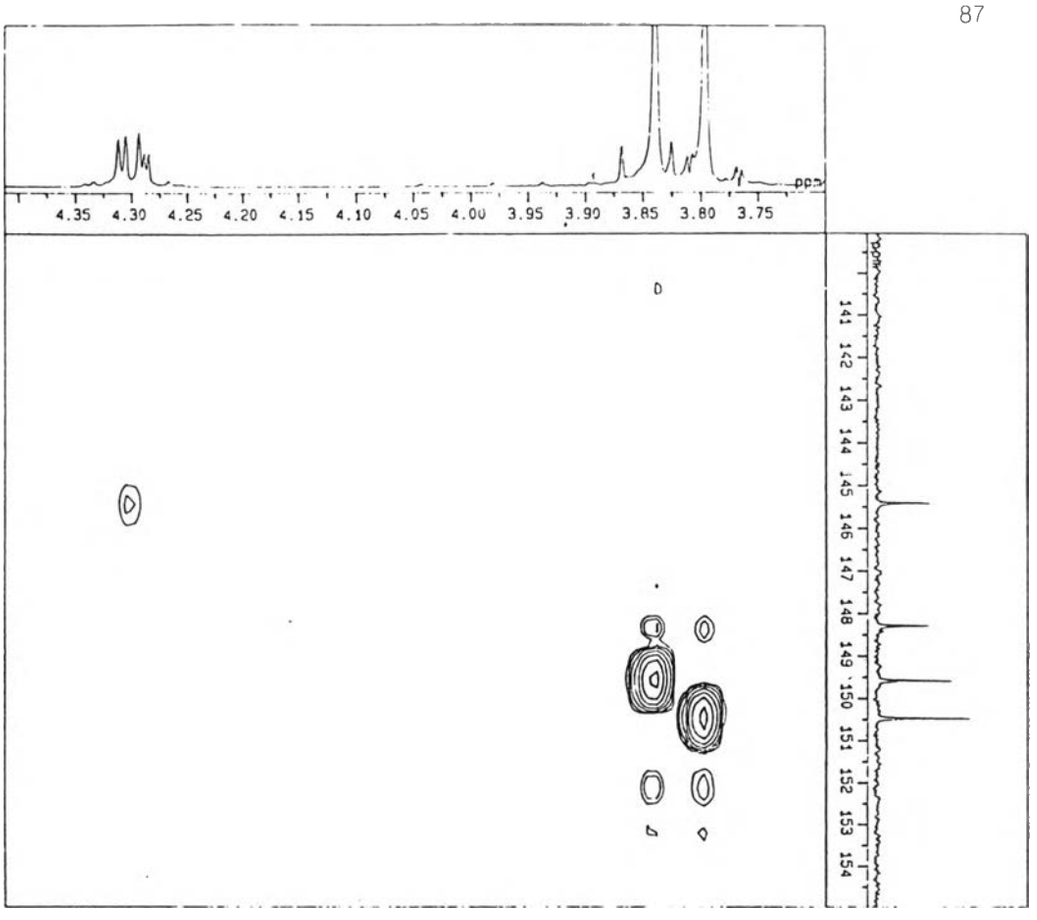


Figure 3.59 HMBC spectrum of compound 5

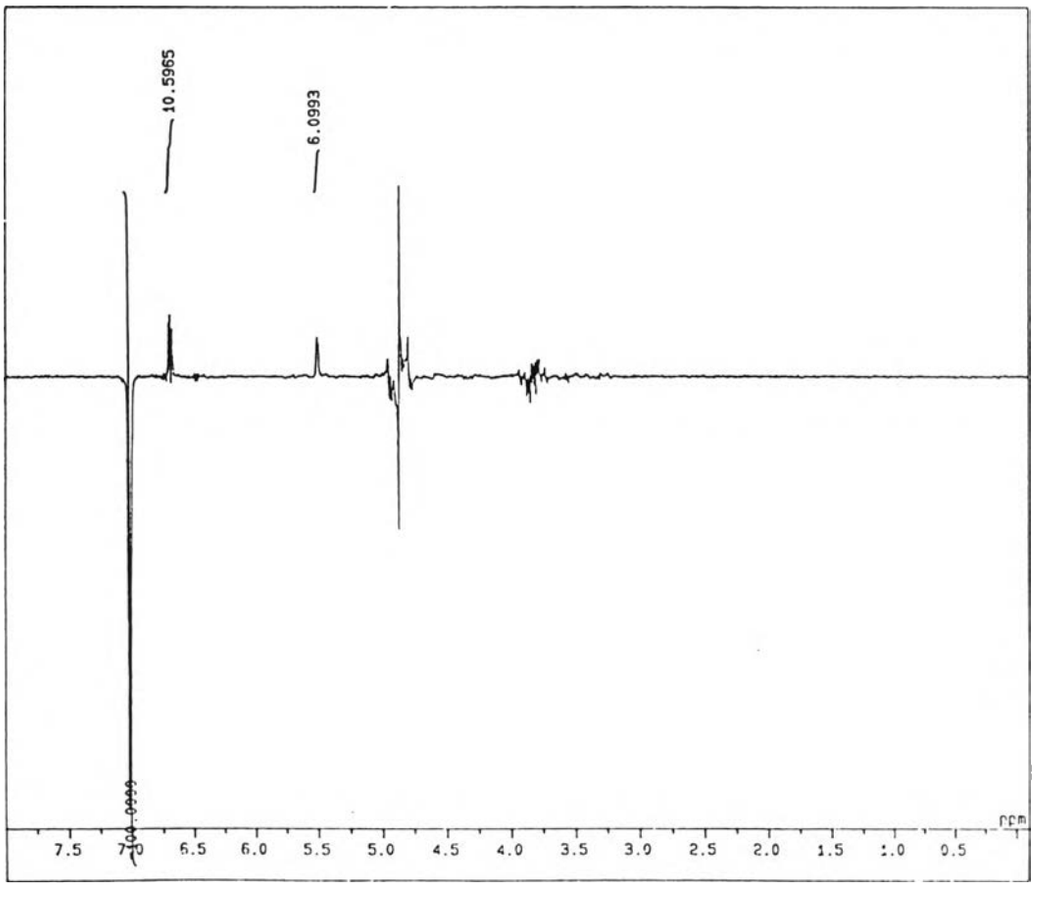


Figure 3.60 NOE DIFF of compound 5 (irradiated at 7.01 ppm)

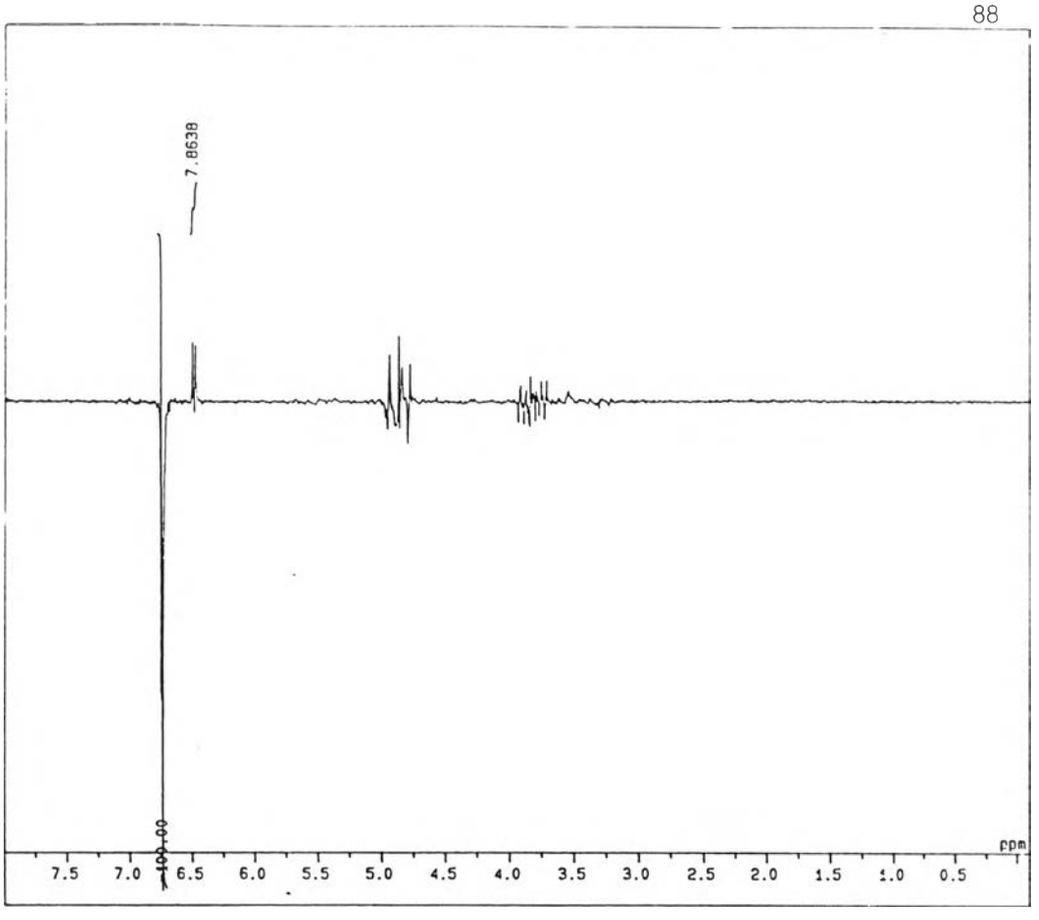


Figure 3.61 NOE DIFF of compound 5 (irradiated at 6.73 ppm)

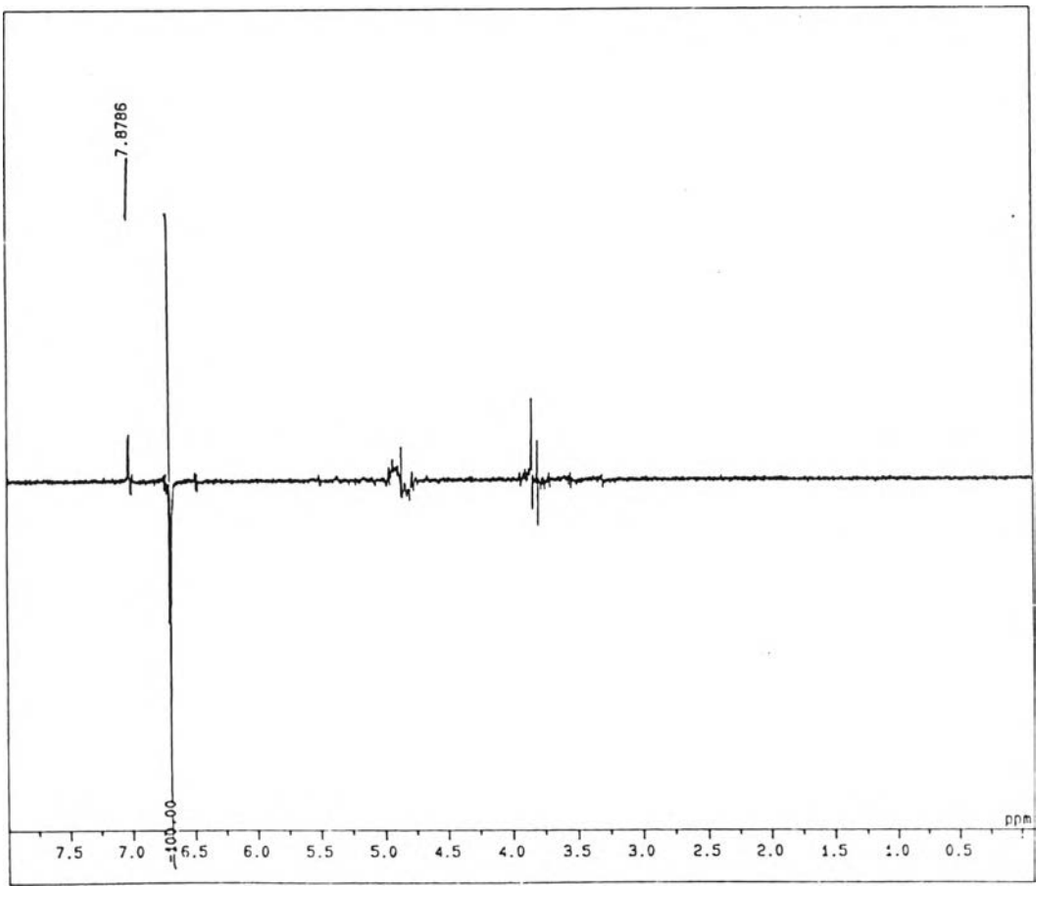


Figure 3.62 NOE DIFF of compound 5 (irradiated at 6.68 ppm)

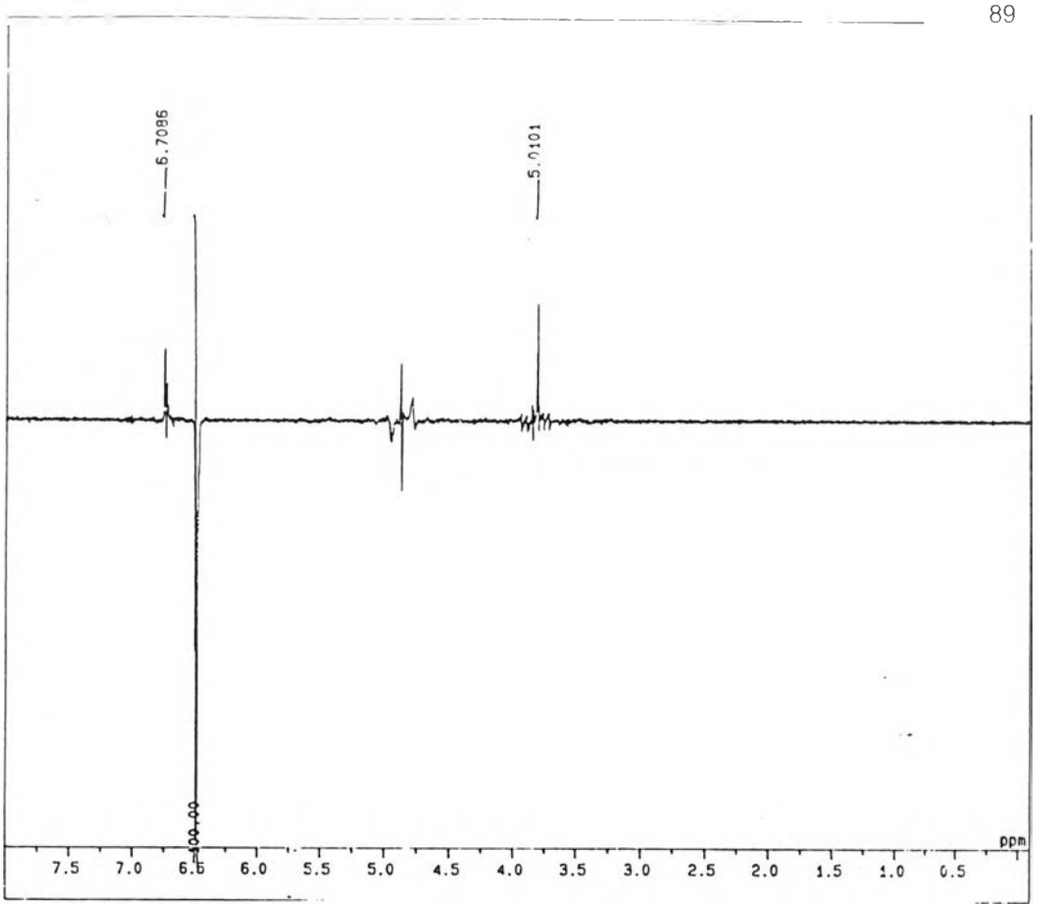


Figure 3.63 NOE DIFF of compound 5 (irradiated at 6.48 ppm)

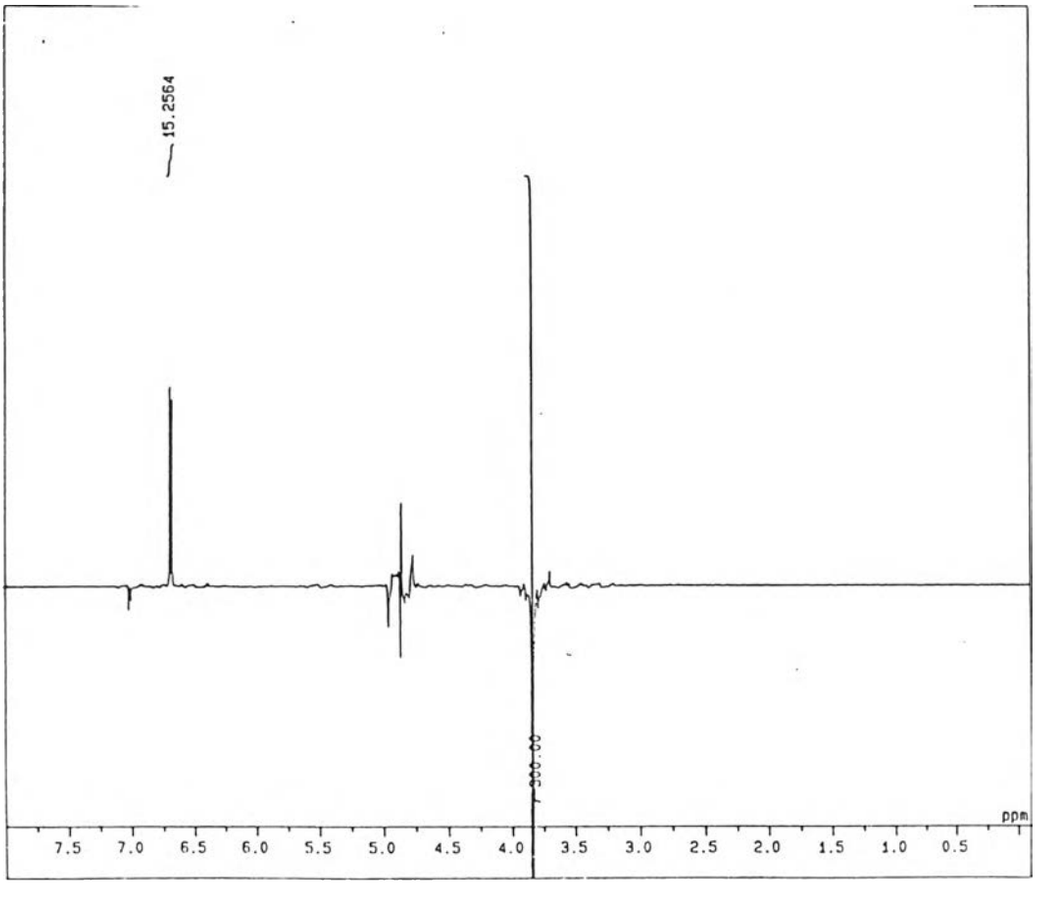


Figure 3.64 NOE DIFF of compound 5 (irradiated at 3.83 ppm)

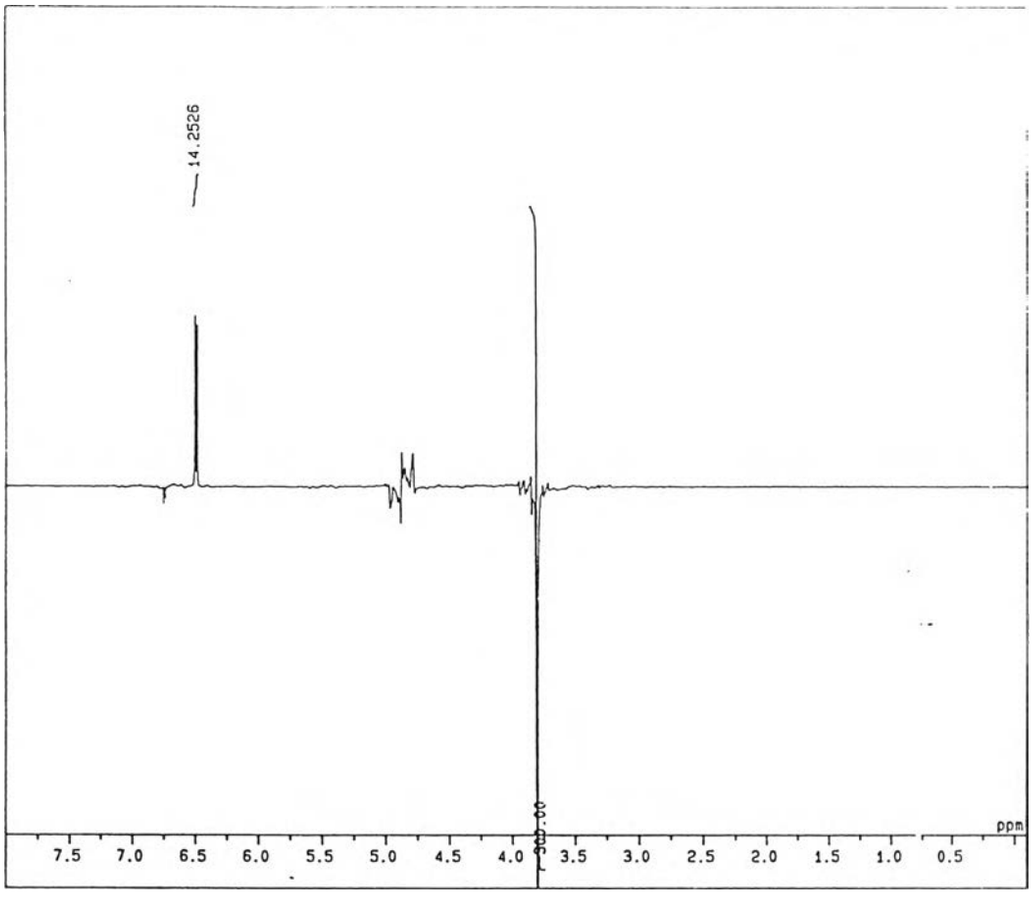


Figure 3.65 NOE DIFF of compound 5 (irradiated at 3.79 ppm)

3.2.6 Compound 6

Compound 6 was isolated as a minor compound of this plant, 10 mg, from crude dichloromethane extract. This compound crystalized as colorless needles, m.p. 246-248 °C (lit[24] 265-267 °C), its Rf value was 0.25 (SiO_2, EtOAc-CH_2Cl_2, 1:9), and it was dissolved in hot methanol, unsoluble in ethyl acetate, dichloromethane and hexane.

The IR absorption bands at 3200-3600 cm^{-1} indicated the presence of hydroxy group, at 1640 cm^{-1} showed the presence of C=O stretching vibration, and at 1600, 1500 and 1460 cm^{-1} displayed the presence of aromatic ring (**Figure 3.66**).

The EI mass spectrum exhibited an [M^+] peak at m/z 268 and peaks of relatively high relative abundance at m/z 254, 253, 136, 132, 117 and 108. The molecular formula, $C_{16}H_{12}O_4$ (**Figure 3.67**), was inferred from the M^+ peak at m/z 268 in the EIMS, and it was supported by ^{13}C and ^1H NMR and DEPT spectra, which showed 16 resonance lines consisting of one methoxy, 8 methines, and 7 quaternary carbons.

The ^1H NMR spectrum (CD_3OD) of this compound showed seven aromatic protons at δ 6.84 (1H, *d*, *J = 2.44 Hz*), 6.93 (1H, *dd*, *J = 8.85, 2.44 Hz*), consisting of ortho protons in A ring, 6.97 (2H, *d*, *J = 8.85 Hz*), 7.46 (2H, *d*, *J = 9.16 Hz*), suggestive of para-substitute of four aromatic protons on B ring, 8.05 (1H, *d*, *J = 8.85 Hz*), and 8.12 (1H, *s*) ppm and methyl protons in methoxy group at δ 3.82 ppm (**Figure 3.68**).

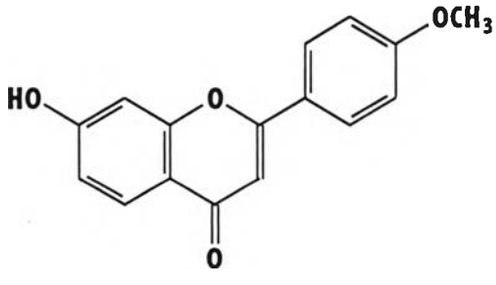
The ^{13}C NMR spectra(**Figure 3.69**) and Dept 90 and 135(**Figure 3.70**) indicated sixteen carbons of fifteen skeleton and one methoxy group(flavonoid characteristic). This compound contained 7 quarternary carbons, 8 methine carbons, and 1 methoxy carbon showed in Table 3.10.

The NOE DIFF experiment was used to confirm the substitution on B ring. The NOE DIFF spectra exhibited the methoxy group substituted at C-4' (**Figure 3.71**).

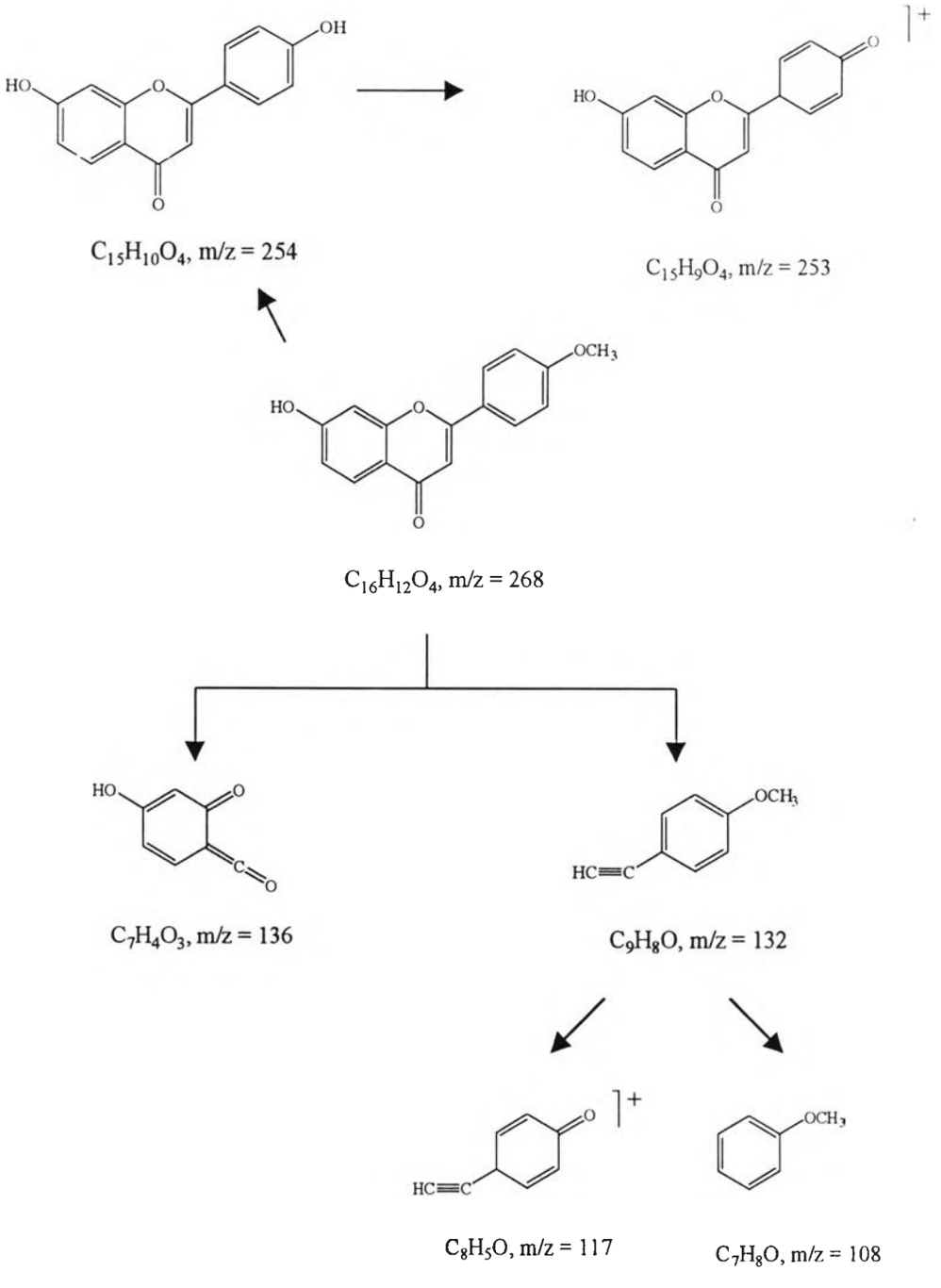
Table 3.10 ^{13}C and ^{1}H NMR data of compound 6

C	Chemical shift (ppm)	
	^{13}C	^{1}H
2	161.1	-
3	103.3	8.12 (*s*,1H)
4	178.0 (C=O)	-
5	154.7	8.85 (*d*, 1H, *J* = *8.85 Hz*)
6	128.4	6.93 (*dd*, 1H, *J* = *8.85, 2.44 Hz*)
7	164.9	-
8	116.6	6.84 (*d*, 1H, *J* = *2.44 Hz*)
9	159.8	-
10	118.1	-
1'	125.6	-
2',6'	131.4	7.46 (*d*, 2H, *J* = *9.16 Hz*)
3',5'	114.9	6.97 (*d*, 2H, *J* = *8.85 Hz*)
4'	125.7	-
-OMe	55.7	3.82 (*s*, 3H)

Based on all spectral data, the structure of compound 6 was elucidated as 7-hydroxy-4'-methoxyflavone.



The structure of compound 6



Scheme 8 Possible mass fragmentation patterns of compound 6

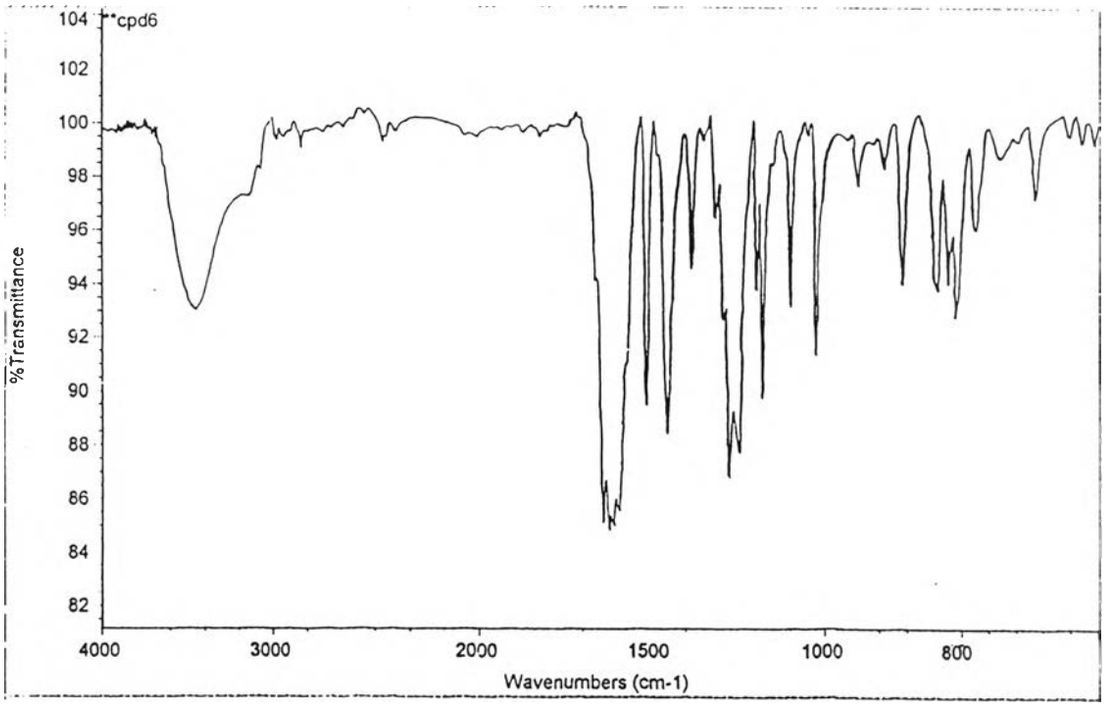


Figure 3.66 IR spectrum of compound 6

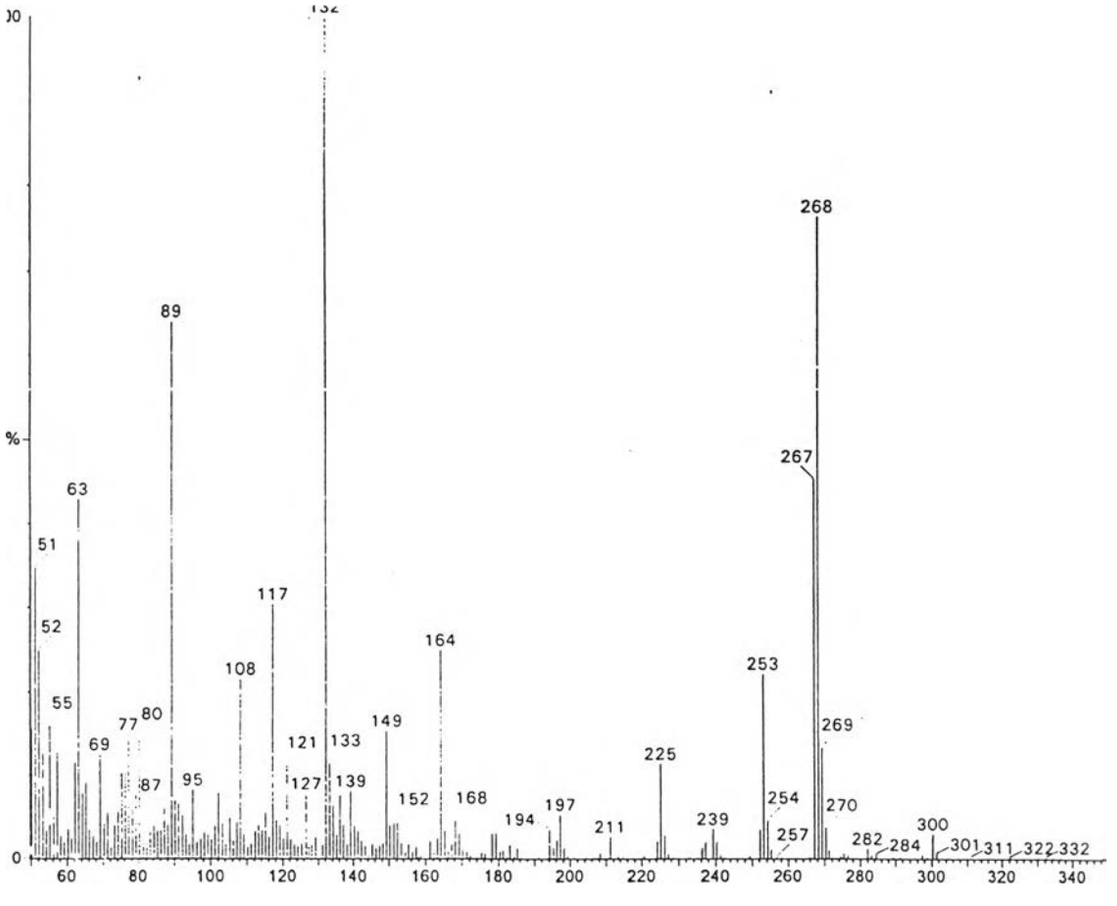


Figure 3.67 Mass spectrum of compound 6

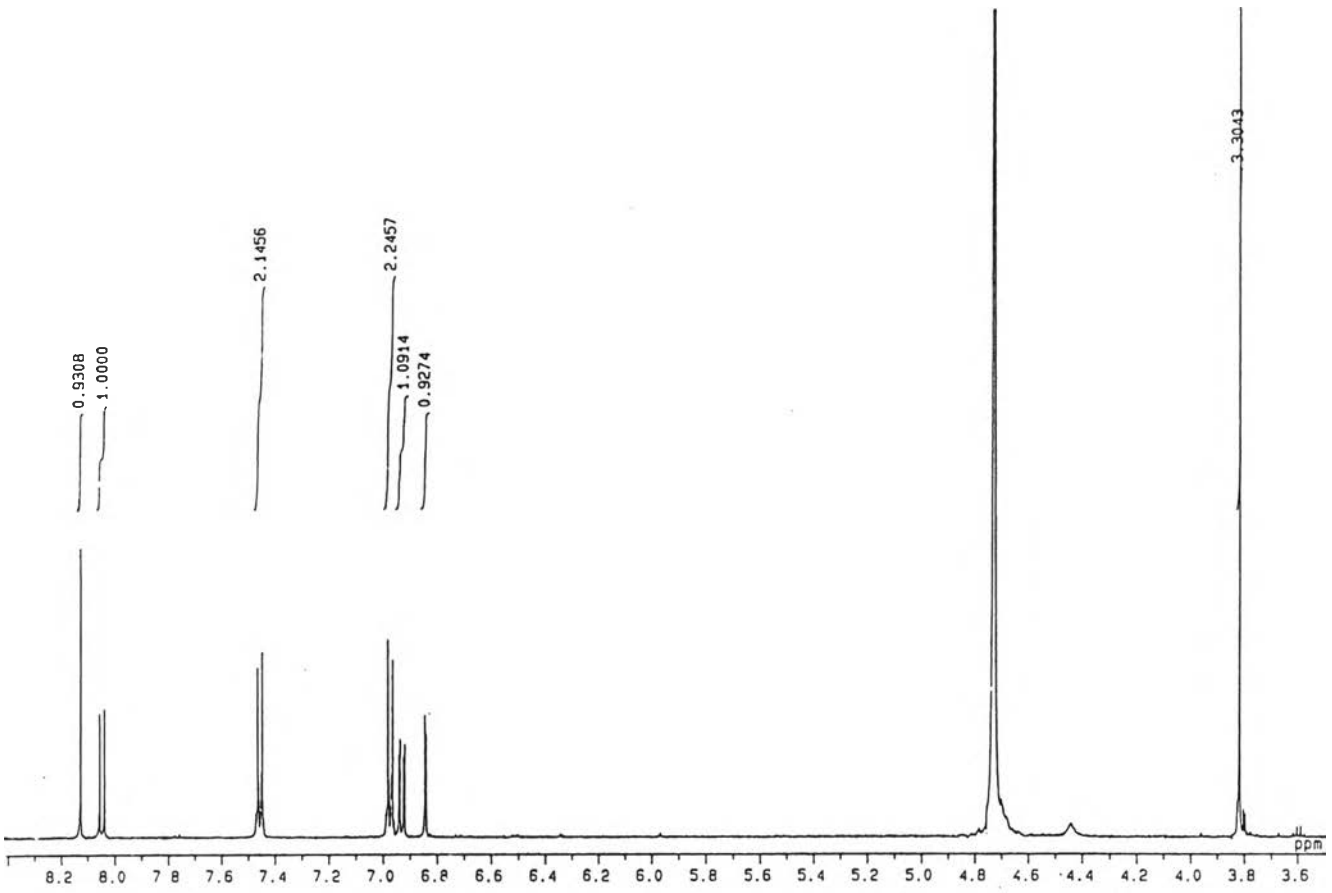


Figure 3.68 ^{1}H-NMR spectrum of compound 6

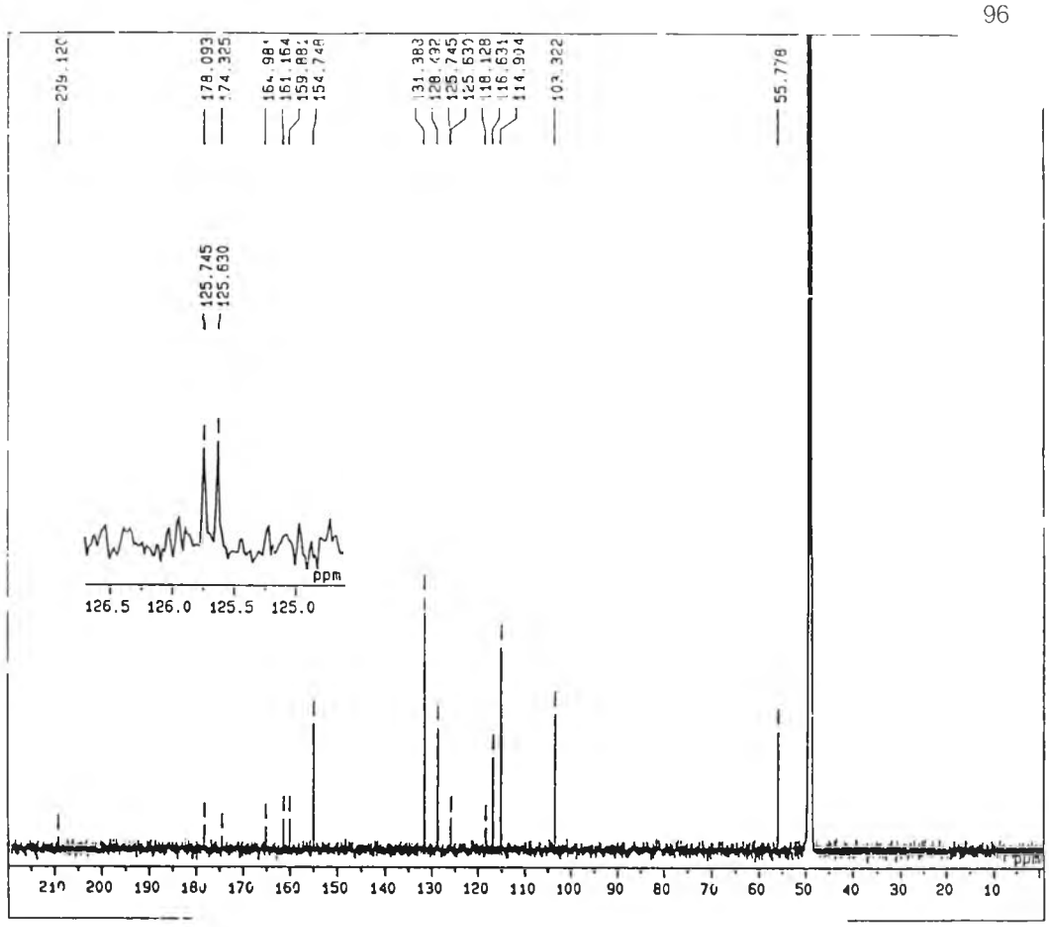


Figure 3.69 ^{13}C-NMR spectrum of compound 6

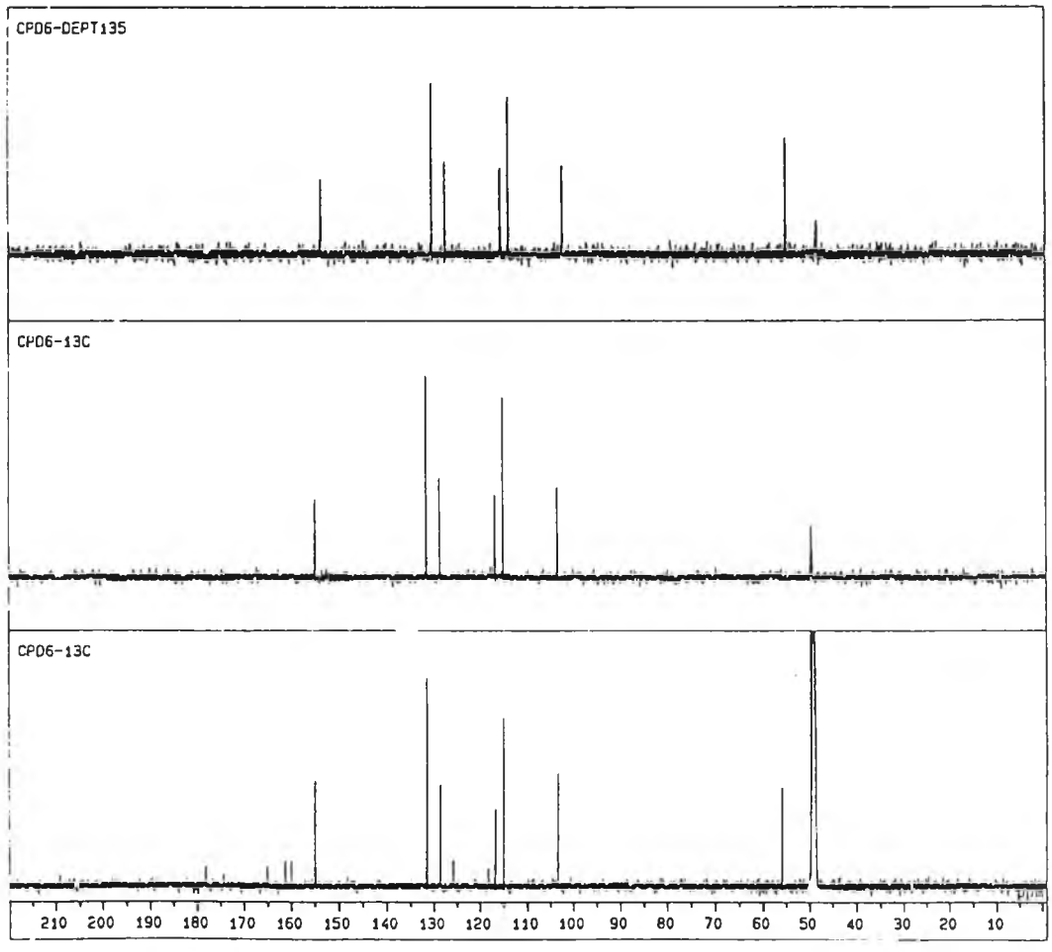


Figure 3.70 DEPT 90 and 135 spectrums of compound 6

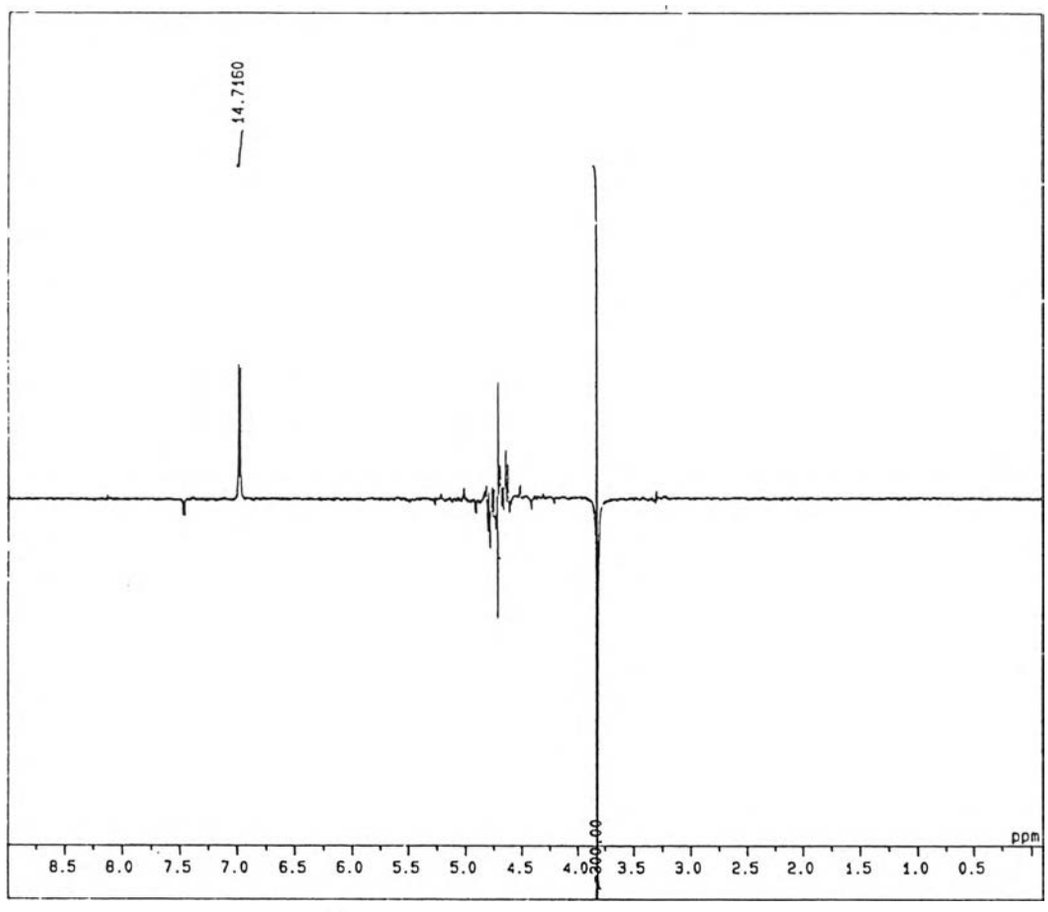


Figure 3.71 NOE DIFF of compound 6 (irradiated at 3.81 ppm)

3.2.7 Compound 7

Compound 7, white crystals, was collected from the hexane crude extract and recrystalized by dichloromethane in methanol. This compound (80 mg) gave the melting point 102-103 °C (lit[27] 109°C), Rf value 0.56 (SiO_2, EtOAc-Heane, 1:9)

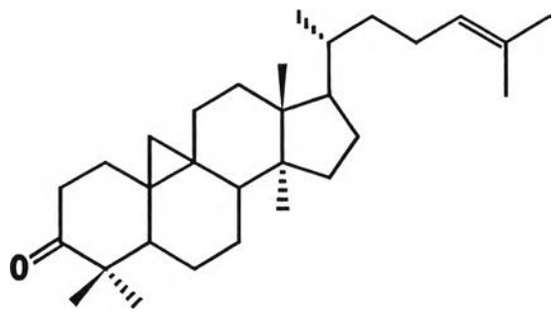
The IR spectrum displayed =C-H stretching vibration at 3050 cm^{-1}, C-H stretching vibration at 2850-2950 cm^{-1}, C=O stretching vibration at 1700 cm^{-1}, and C=C stretching vibration at 1660 cm^{-1}, CH_2 and CH_3 bending vibration at 1475 and 1380 cm^{-1}, and cyclopropane showed bending vibration at 1460-1440 cm^{-1} (**Figure 3.72**).

The mass spectrum was observed the molecular ion at m/z 424 and other fragments were at m/z 409 (M^+-CH_3), 381 (M^+-C_3H_7), 355 (M^+-C_5H_9), 313 (M^+-C_8H_{15}) (**Figure 3.73**). The molecular formula of compound 7 was confirmed as $C_{30}H_{48}O$ from the EIMS and NMR data.

The ^1H NMR spectra ($CDCl_3$, 500 MHz, δ-scale) of compound 7 (**Figure 3.74**) showed a pair of doublets centred at 0.55 and 0.76 ppm (lit[27] 0.58 and 0.81, respectively) attributable to the non-equivalent methylene protons of a cyclopropane ring, the proton signals of seven methyl groups at δ 0.86, 0.88, 0.97, 1.02, 1.07, 1.58, 1.66 ppm and the remaining methine and methylene proton signals of triterpenoid from 0.90 to 5.10 ppm. The proton positions were vague.

The ^{13}C NMR spectrum (**Figure 3.75**) revealed 30 carbons of triterpenoid skeleton, Dept 90 and 135 (**Figure 3.76**) indicated 7 quarternary carbons, 5 methine carbons, 11 methylene carbons and 7 methyl carbons. The carbons position of compound 7 (cycloartenone) (**Table 3.11**) compared with cycloart-24-ene-3β,28-diol (**7a**), and 21,24 (RS)-dihydroxycycloart-25-en-3-one (**7b**).

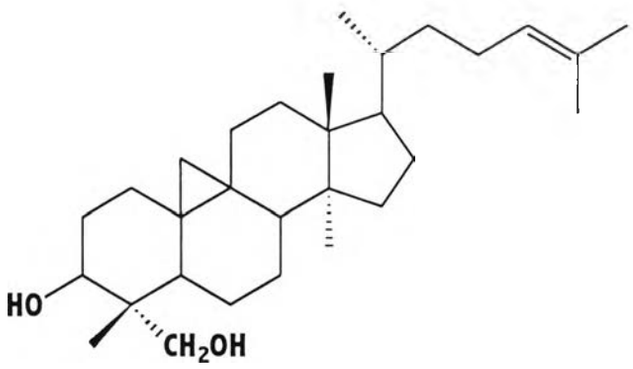
From all data, the structure of compound 7 was elucidateded as cycloartenone.



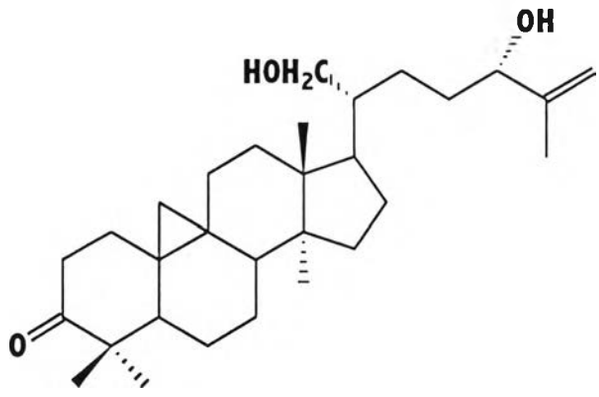
The structure of compound 7

Table 3.11 ^{13}C NMR of compound 7 compared with cycloart-24-ene-3β,28-diol (7a) and 21,24 (RS)-dihydroxycycloart-25-en-3-one (7b)[28].

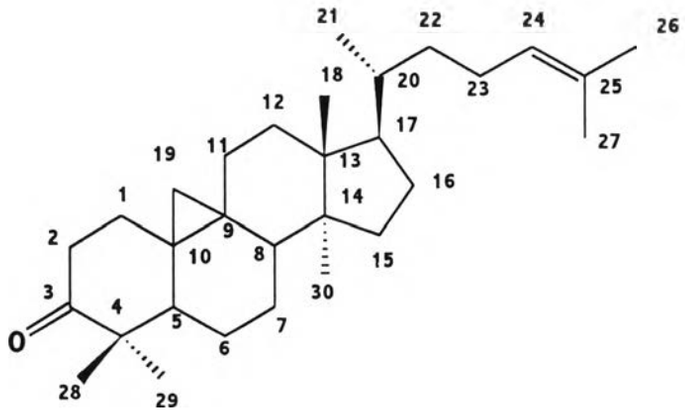
Position	Compound 7	7a	7b
1	***33.4***	31.7	***33.4***
2	***37.4***	30.2	***37.4***
3	***216.5***	77.0	***216.5***
4	***50.2***	43.7	***50.2***
5	***48.4***	42.5	***48.4***
6	***21.4***	21.0	***21.4***
7	***25.8***	25.7	***25.8***
8	***47.8***	47.9	***47.8***
9	***21.0***	20.0	***21.0***
10	***25.9***	25.4	***26.0***
11	***26.7***	26.4	***26.6***
12	***32.7***	32.9	***32.1***
13	***45.2***	45.2	***45.1***
14	***48.7***	48.8	***48.8***
15	***35.5***	35.6	***35.4***
16	***28.1***	28.1	***27.5***
17	***52.2***	52.3	***46.4***
18	***18.0***	18.0	***18.3***
19	***29.5***	30.0	***29.5***
20	***35.8***	***35.9***	42.4
21	***18.2***	***18.2***	62.5
22	***36.3***	***36.3***	25.0
23	***24.9***	***24.9***	30.7
24	***125.1***	***125.3***	76.2
25	***130.9***	***130.9***	147.6
26	***17.6***	***17.6***	110.9
27	***25.7***	***25.7***	17.7
28	***22.1***	71.1	***22.2***
29	***20.7***	10.1	***20.7***
30	***19.2***	19.3	***19.4***



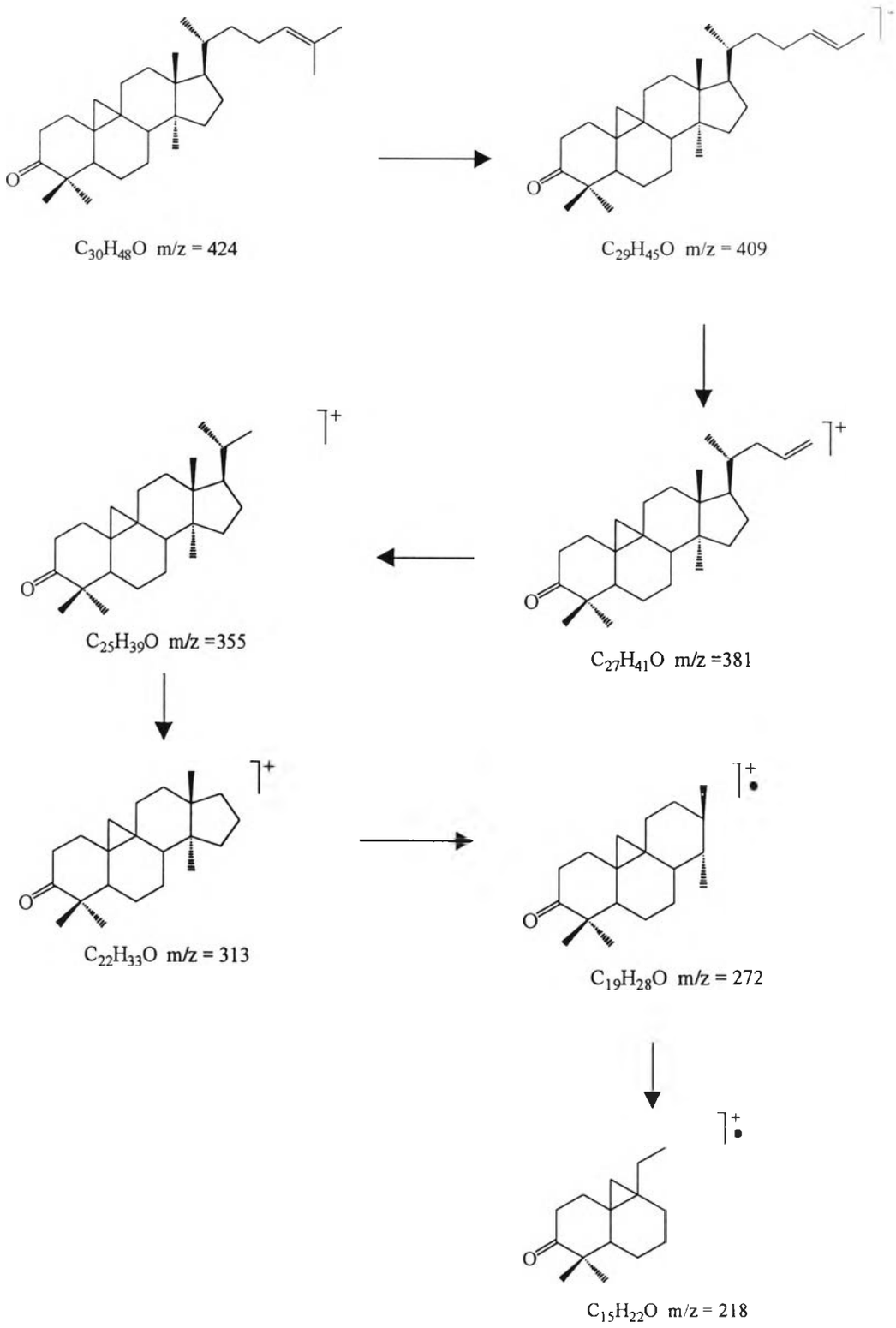
Structure of cycloart-24-ene-3β,28-diol (7a)[28]



Structure of 21,24(RS)-dihydroxycycloart-25-en-3-one (7b)[28]



Structure of cycloartenone (compound 7)



Scheme 9 Possible fragmentation patterns of compound 7

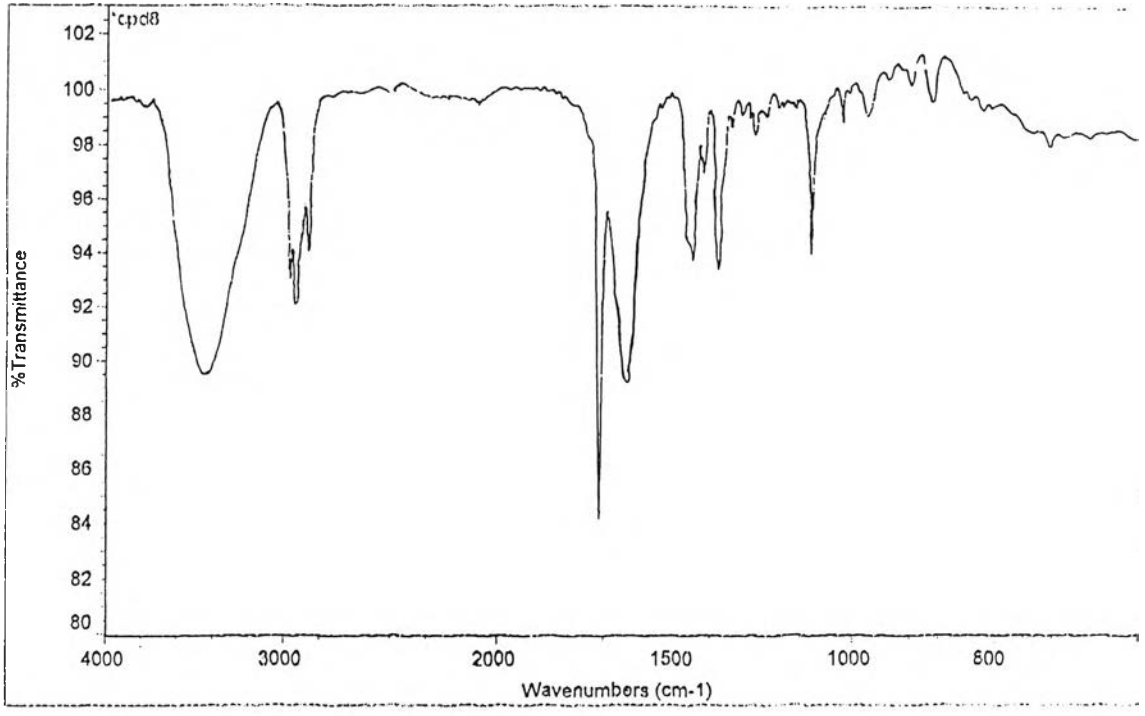


Figure 3.72 IR spectrum of compound 7

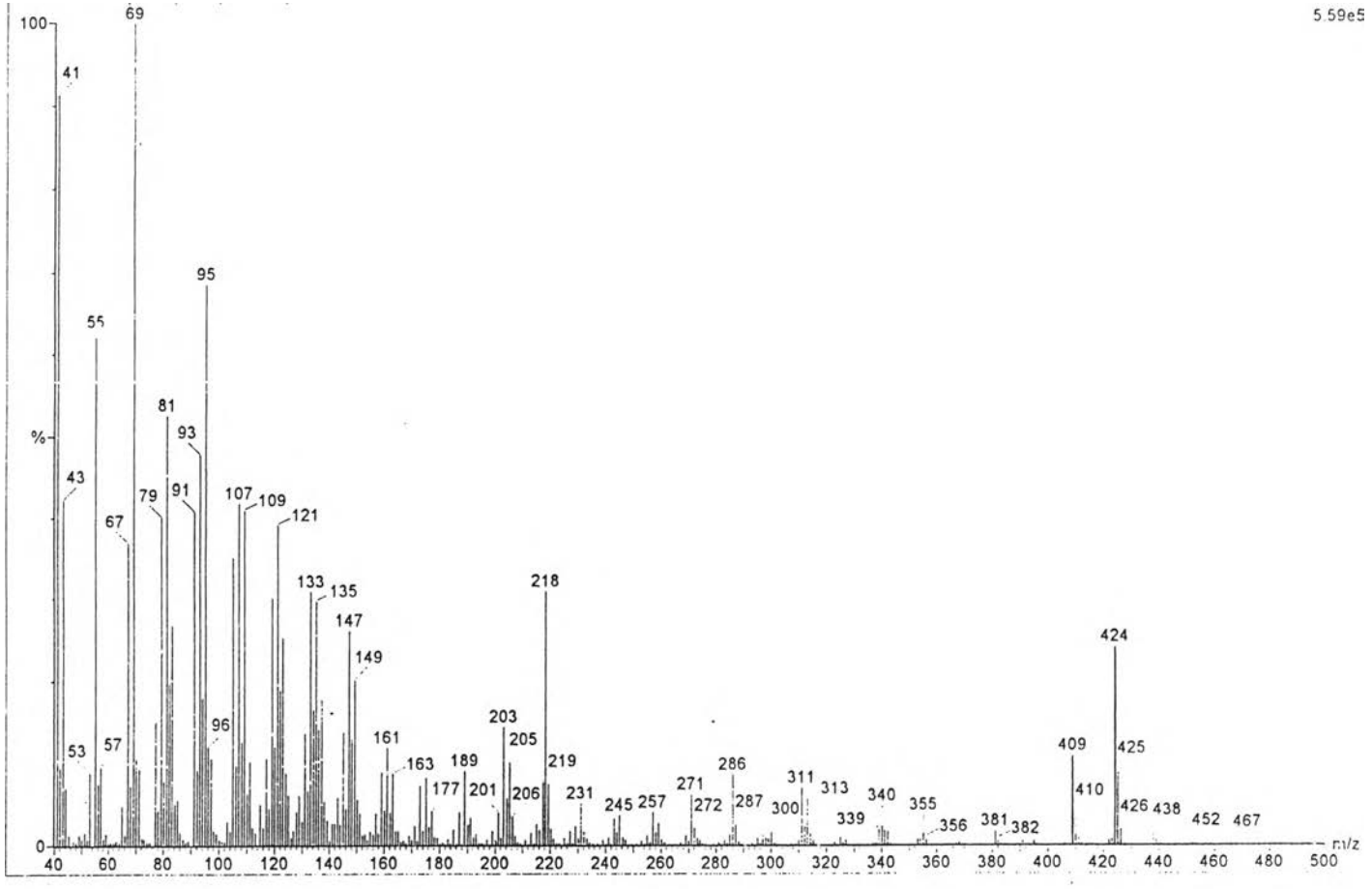


Figure 3.73 Mass spectrum of compound 7

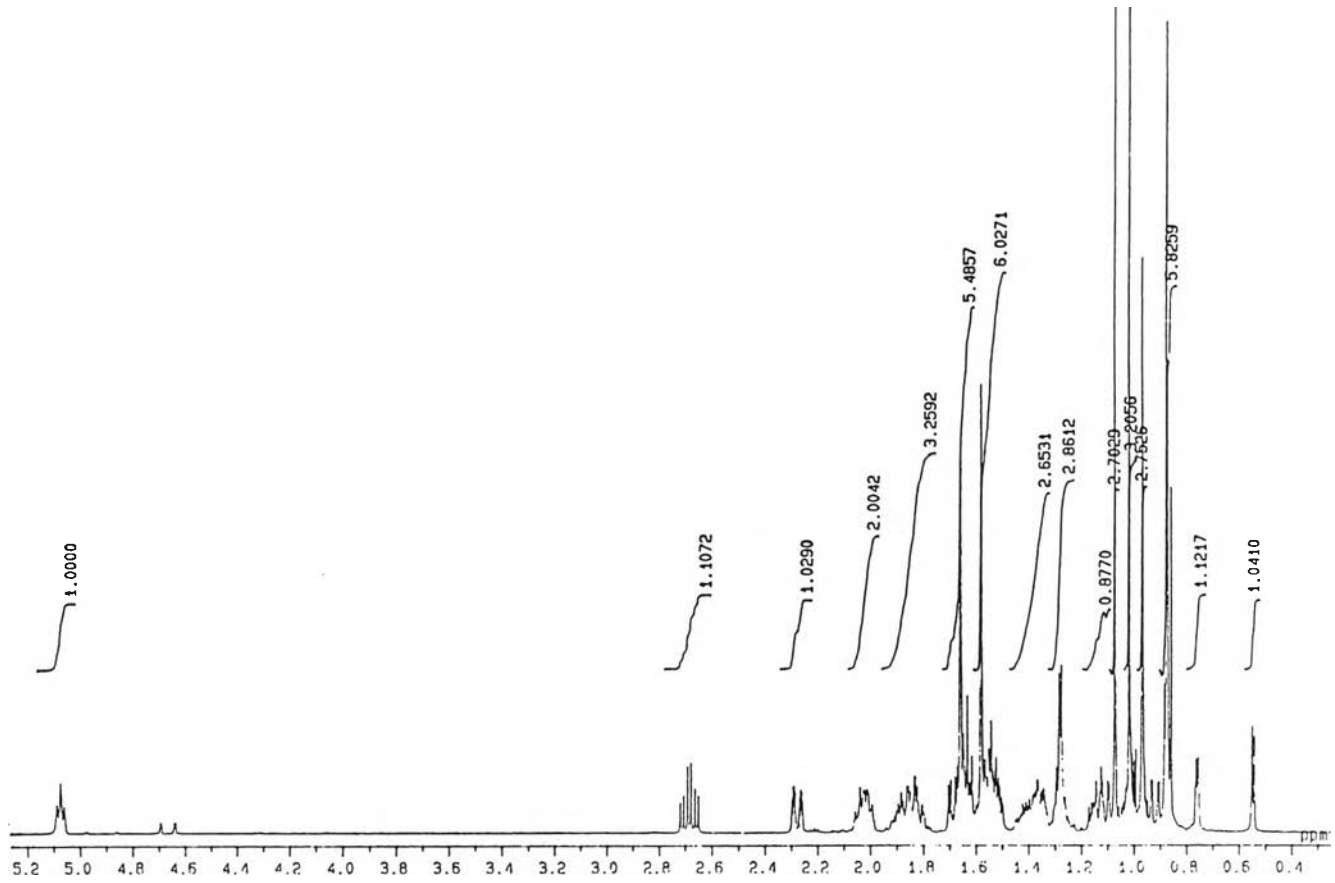


Figure 3.74 ^{1}H-NMR spectrum of compound 7

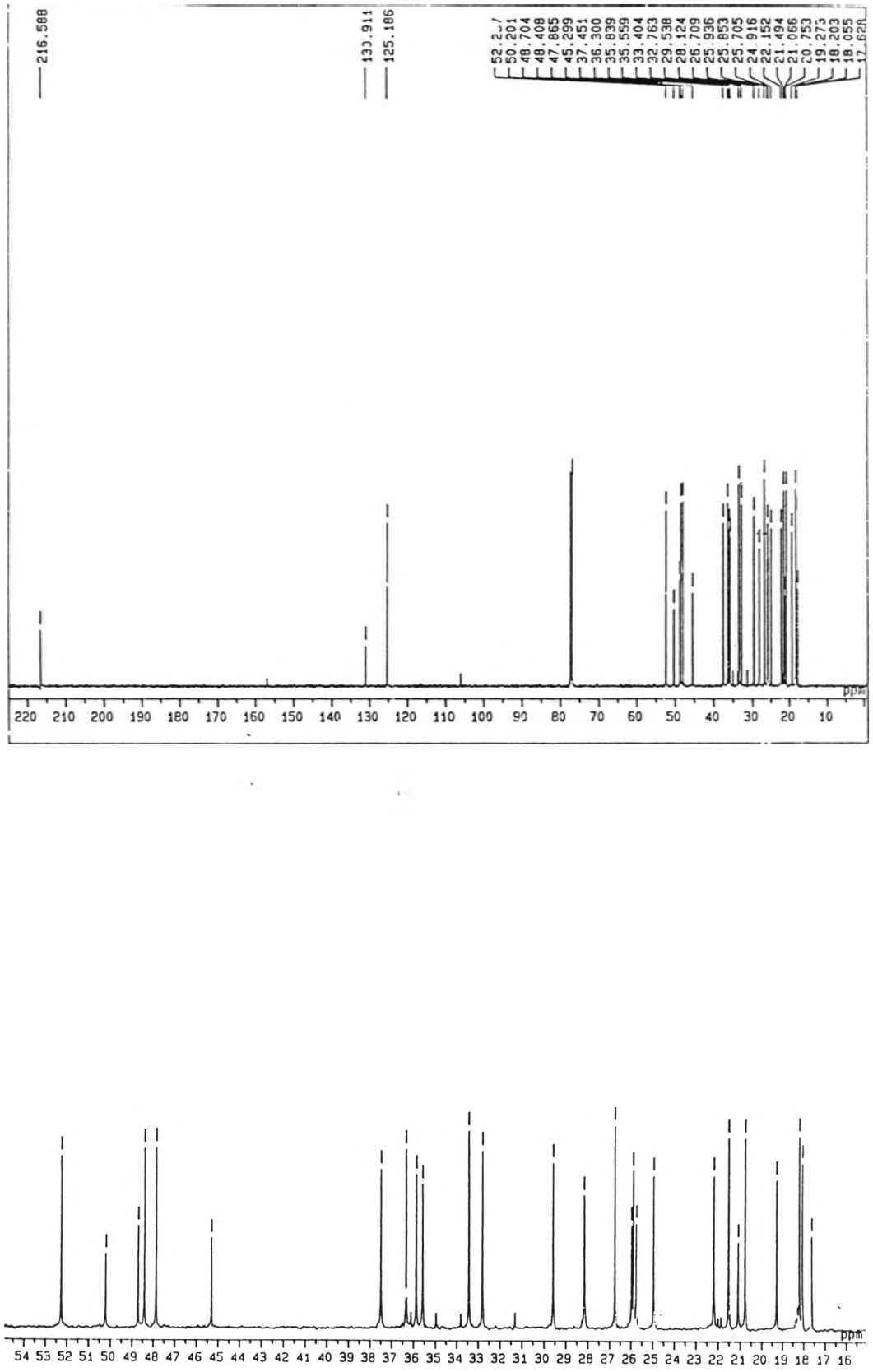


Figure 3.75 ^{13}C-NMR spectrum of compound 7

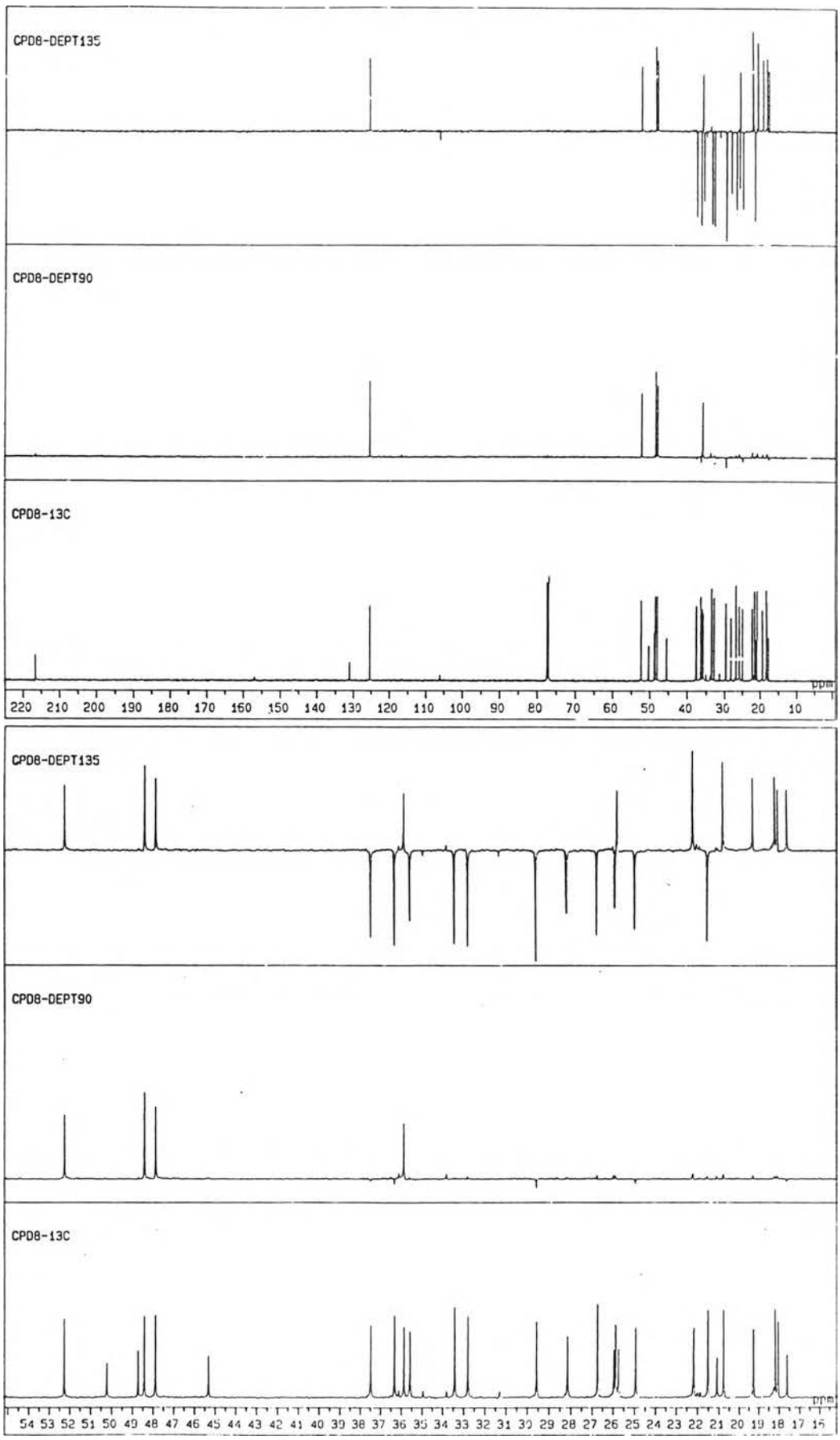


Figure 3.76 DEPT 90 and 135 spectrums of compound 7

3.3 The Biological Activity of Isolated Compounds from *C. cochinchinensis*

The antibacterial activity, in-vitro IC_{50} values of KB cell lines, free radicals scavenging activity, and % NBT reduction inhibition of isolated compounds from *C. cochinchinensis* were shown in Table 3.12, 3.13, 3.16 and 3.17, respectively.

Table 3.12 Antibacterial Activity of Isolated Compounds from *C. cochinchinensis*.

Compound	*E.coli*	*S.aureus*	*B.cereus*	*S.derby*	*L.mono*	FS
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	6	6	0	0	8
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	-	-	-	-	-	-
steptomycin	14	18	12	15	0	18

Table 3.13 Cytotoxic Activity Against KB Cell Lines by MTT Assay*

Samples	IC_{50} (μg/ml) λ_{550} nm
1	1.8
2	9
3	3.1
4	3.5
5	10
6	-
7	>10 (~32 μg/ml)

*performed at Natural Products Research Section, Research Division, National Cancer Institute, Thailand

Table 3.14 Free Radicals Scavenging Activity of Isolated Compounds on DPPH (0.25 mM)

Sample	Conc (mM)	% radical scavenging
Compound 1	1.000	31.47
	0.500	30.57
	0.250	30.38
	0.125	27.74
Compound 2	1.000	85.96
	0.500	73.39
	0.250	61.65
	0.125	49.73
	0.100	45.50
	0.075	40.51
Compound 3	1.000	33.03
	0.500	30.08
	0250	29.26
	0.125	27.92
Compound 4	1.000	4.19
	0.500	3.40
	0.250	3.46
	0.125	3.19
Compound 5	1.000	95.12
	0.500	94.20
	0.250	90.50
	0.125	75.55
	0.100	62.03
	0.075	52.70
	0.050	39.32
Compound 6	-	-
Compound 7	-	-

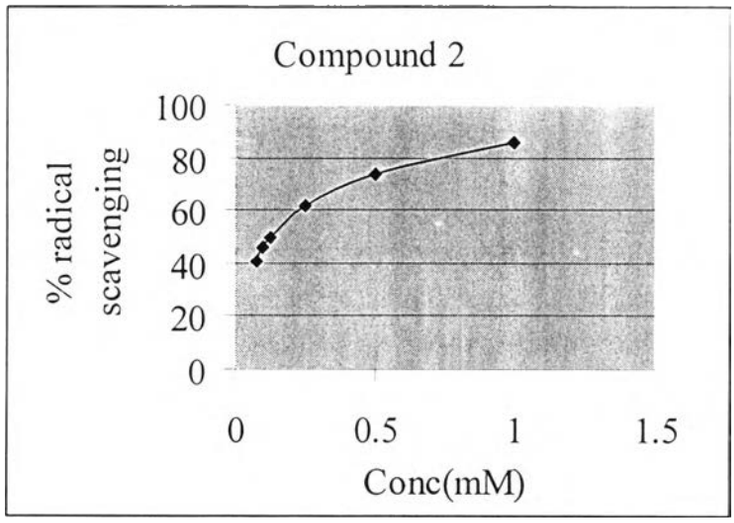


Figure 3.77 Scavenging effect of compound 2

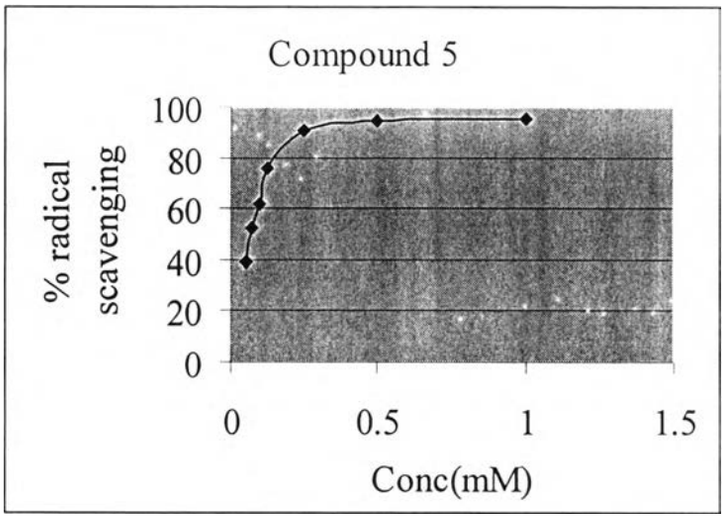


Figure 3.78 Scavenging effect of compound 5

Table 3.15 Free Radicals Scavenging Activity of BHA on DPPH (0.25 mM)

Sample	Conc (mM)	% radical scavenging
BHA*	1.000	96.19
	0.500	94.18
	0.200	70.74
	0.100	44.71
	0.050	21.93
	0.025	11.18

BHA = Butylated Hydroxyanisole

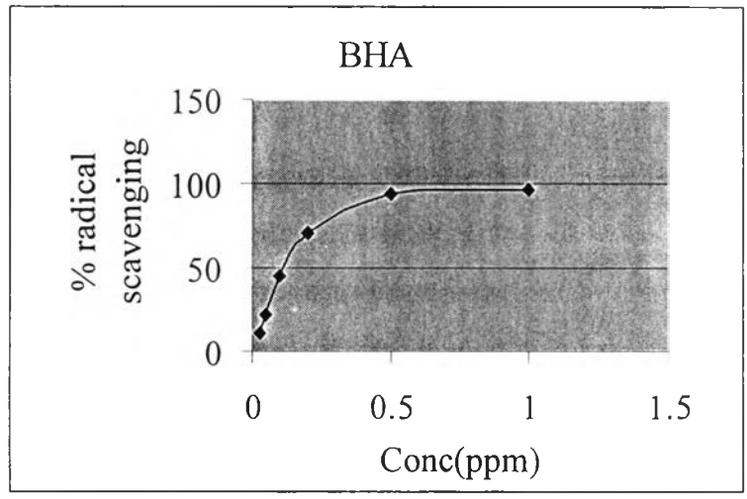


Figure 3.79 Scavenging effect of BHA

Table 3.16 IC_{50} on Free Radicals Scavenging Activity of Isolated Compounds on DPPH

Compound	IC_{50} (mM)
1	>1.0000
2	0.1388
3	>1.0000
4	>1.0000
5	0.0612
6	-
7	-
BHA	0.1177

Table 3.17 Xanthine Oxidase Activity of Isolated Compounds on Xanthine-Xanthine Oxidase System

Compound	% NBT reduction inhibition at 500 μM
1	6.08
2	12.69
3	6.90
4	4.76
5	29.60
6	11.46
7	-
Allopurinol*	34.65

Allopurinol = oral drug to treat gout

In terms of biological activities, Compound 2 and 5 showed high free radicals scavenging activity on DPPH (IC_{50} 138.8, 61.2 μM, respectively), while these two compounds showed only moderate in-vitro cytotoxicity against KB cell lines. Compound 1, 3, and 4 were also found to be high in-vitro cytotoxicity against KB cell lines.

The biological activity data above provided that the radical scavenging activity depends on the structure and the substituents on B ring. The presence of 7-hydroxy group on ring A seemed to be less important. From the literature review[5], the major determinants for radicals scavenging capacity are (i) the presence of a catechol group in ring B, which has the better electron-donating properties and is a radical target, and (ii) a 2,3 double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. Our data also supported these approaches.

Compound 5 (new compound) also showed highest %NBT reduction inhibition (29.60) among the isolated compound from this plant and closed to allopurinol (34.65) which used as oral drug to treat gout. Compound 2 and 6 showed moderate %NBT reduction inhibition (12.69, 11.46).