CHAPTER 3

RESULTS AND DISCUSSION

3.1 Primary Bioassay Screening Results of Crude Extracts.

3.1.1 Antioxidant test

Free radical scavenging activity on DPPH assay (qualitative : TLC autograph and quantitative : spectrophotometer) were used for primary screening test for antioxidants of all crude extracts from *Dalbergia cochinchinensis* .

Table 3.1 Qualitative antioxidant results from TLC autographic assay of various crude extracts.

Crude extracts	Antioxidant
Hexanes	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	-

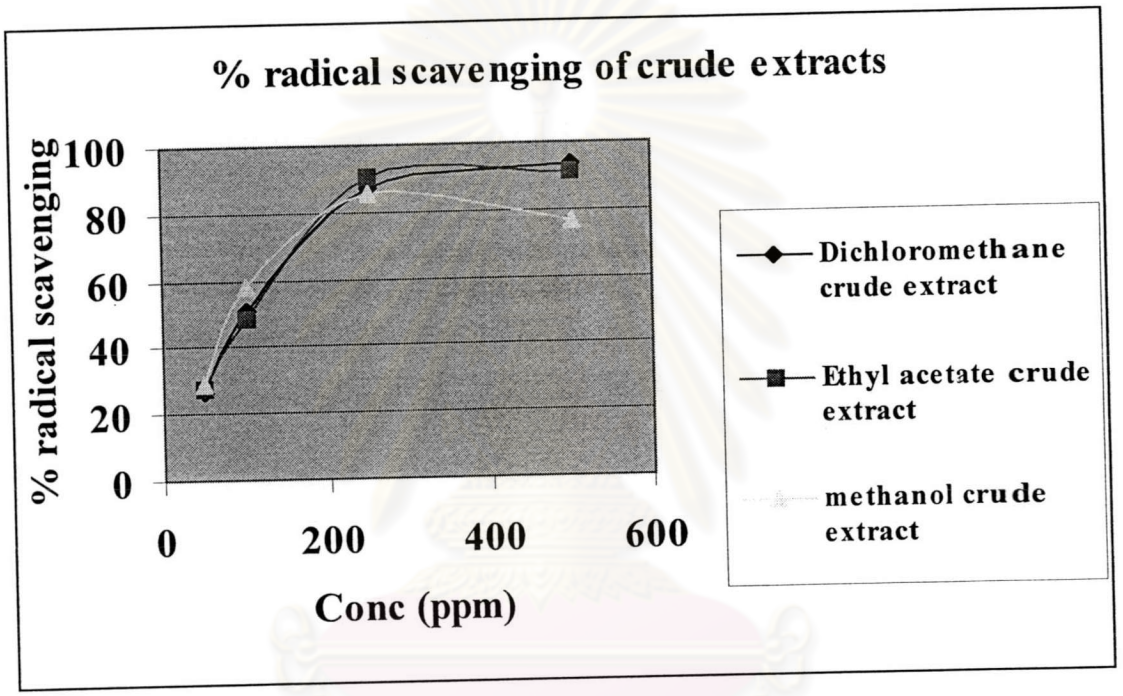


Figure 3.1 Scavenging effect of crude extracts

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

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

NT* = not tested

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

3.2 Properties and Structure Elucidation of Isolated Compounds

3.2.1 Compound 1

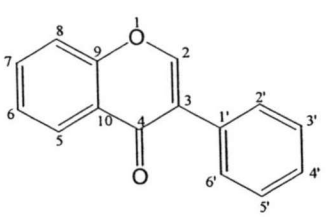
Compound **1** was separated by column chromatography of ethyl acetate extract and was obtained as white needle crystals (0.50 g) with mp. 195-198 °C, R_f 0.38 (SiO_2, EtOAc-CH_2Cl_2, 2:8). It was soluble in acetone and slightly in methanol but not in hexane, dichloromethane and ethyl acetate.

The IR spectrum (**Figure 3.2**) exhibited strong absorption bands of a carbonyl group which conjugated with aromatic rings and also hydrogen-bonded with a hydroxyl group (1644 cm^{-1}), aromatic ring (1511 cm^{-1}), a hydroxyl group (3257 cm^{-1}) and chelated hydroxyl (3441 cm^{-1}).

The mass spectrum (**Figure 3.3**) of compound **1** showed a molecular ion peak, $[M^+]$, at m/z 270 along with fragment ion peaks at m/z 153 and 118 in the electron impact mass spectrum (EIMS). From EIMS, ^1H and ^{13}C-NMR data, compound **1** contained molecular formula $C_{15}H_{10}O_5$, indicating 11 degrees of unsaturation.

The ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.4**) of compound **1** showed signals for six aromatic protons. Four two-proton-doublets ortho coupling to each other at δ 7.46 and 6.90 (d, J = 8.1 Hz) were due to H-2', H-6' and H-3', H-5' of ring B. Two one-proton doublets meta coupling to each other at δ 6.41 and 6.27 (d, J = 2.1 Hz) were assigned to H-6 and H-8 of ring A. One proton attached to the sp^2 carbon at δ 8.16 (1H, s). This means that compound **1** should be an isoflavone or a flavone derivative.

A total of fifteen carbons appeared in the ^{13}C-NMR spectrum (**Figure 3.5**), which indicated seven methine carbons (94.4, 99.7, 115.9, 115.9, 131.0, 131.0 and 154.2), and eight quaternary carbons (105.2, 123.0, 123.9, 156.7, 158.4, 163.8, 165.0 and 181.5 . From the evidence of ^1H and ^{13}C-NMR spectrum, compound **1** was deduced to be basic skeleton isoflavone derivative. The proton and carbon assignments of compound **1** were shown in **Table 3.4**. This structure was also confirmed by comparison with reported data of genistein in **Table 3.5**.



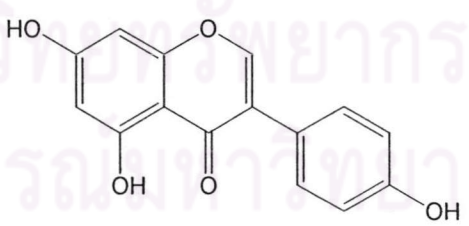
Isoflavone skeleton

Table 3.4 ^{1}H and ^{13}C spectral data of compound **1**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	154.2	8.16	1H, s
3	123.9		
4	181.5		
5	163.8		
6	99.7	6.41	1H, d, J = 2.1 Hz
7	165.0		
8	94.4	6.27	1H, d, J = 2.1 Hz
9	156.7		
10	105.2		
1'	123.0		
2'	131.0	7.46	2H, d, J = 8.1 Hz
3'	115.9	6.90	2H, d, J = 8.1 Hz
4'	158.4		
5'	115.9	6.90	2H, d, J = 8.1 Hz
6'	131.0	7.46	2H, d, J = 8.1 Hz

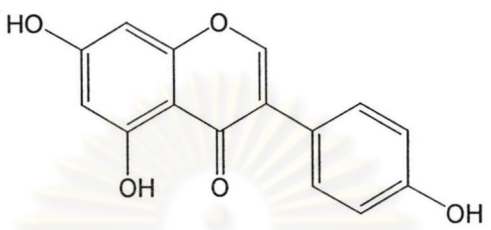
Table 3.5 ^{13}C NMR data of compound **1** compared with Genistein.[21]

Position	Compound **1**	Genistein
2	154.2	153.6
3	123.9	121.4
4	181.5	180.2
5	163.8	157.6
6	99.7	98.6
7	165.0	164.3
8	94.4	93.7
9	156.7	157.6
10	105.2	104.6
1'	123.0	122.4
2'	131.0	130.0
3'	115.9	115.2
4'	158.4	162.1
5'	115.9	115.2
6'	131.0	130.0

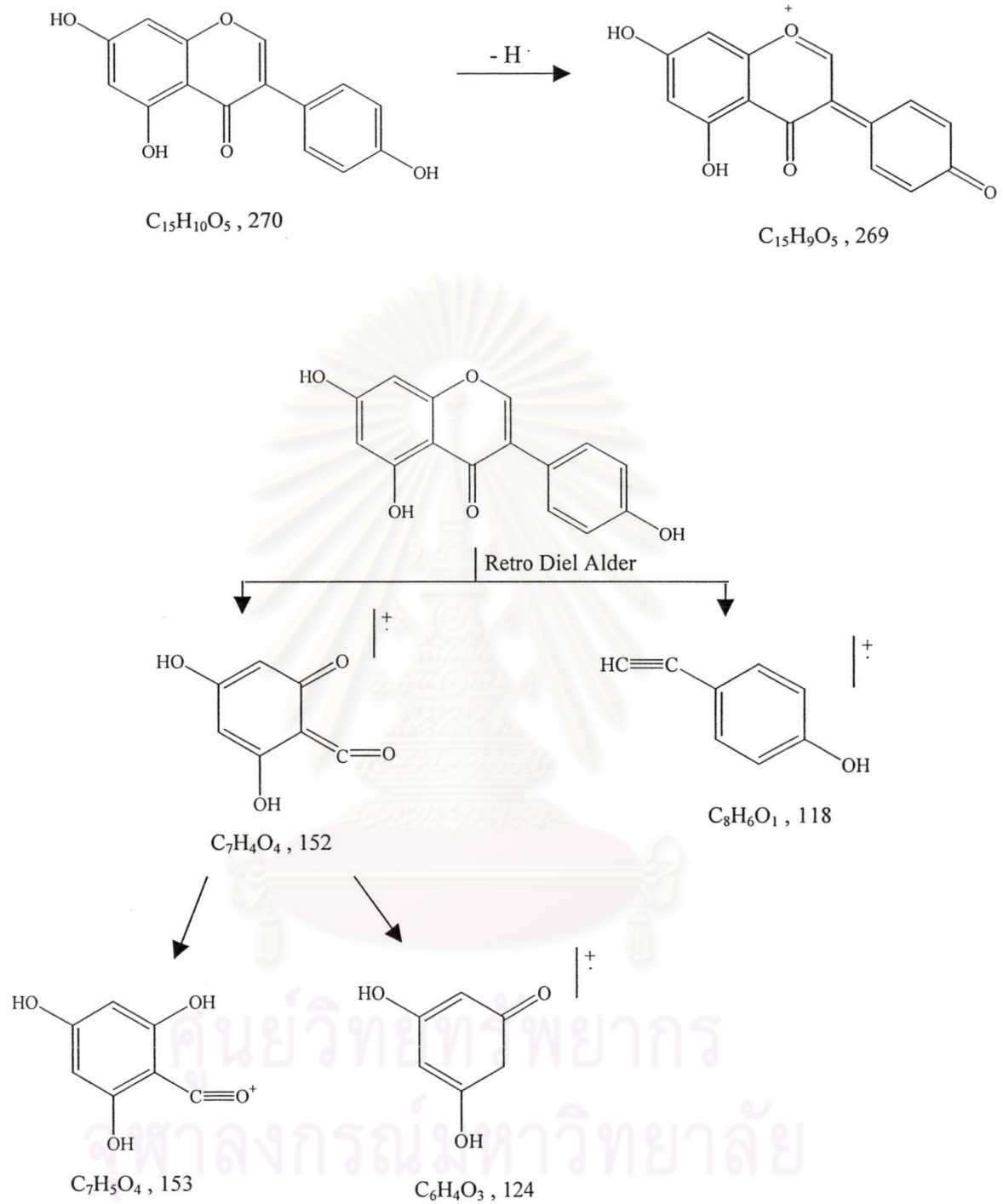


Genistein

Based on the above spectroscopic data, compound **1** was established as 5,7,4'-trihydroxyisoflavone or genistein.



Structure of compound **1** (genistein)



Scheme 3.1 Possible mass fragmentation patterns of compound **1**

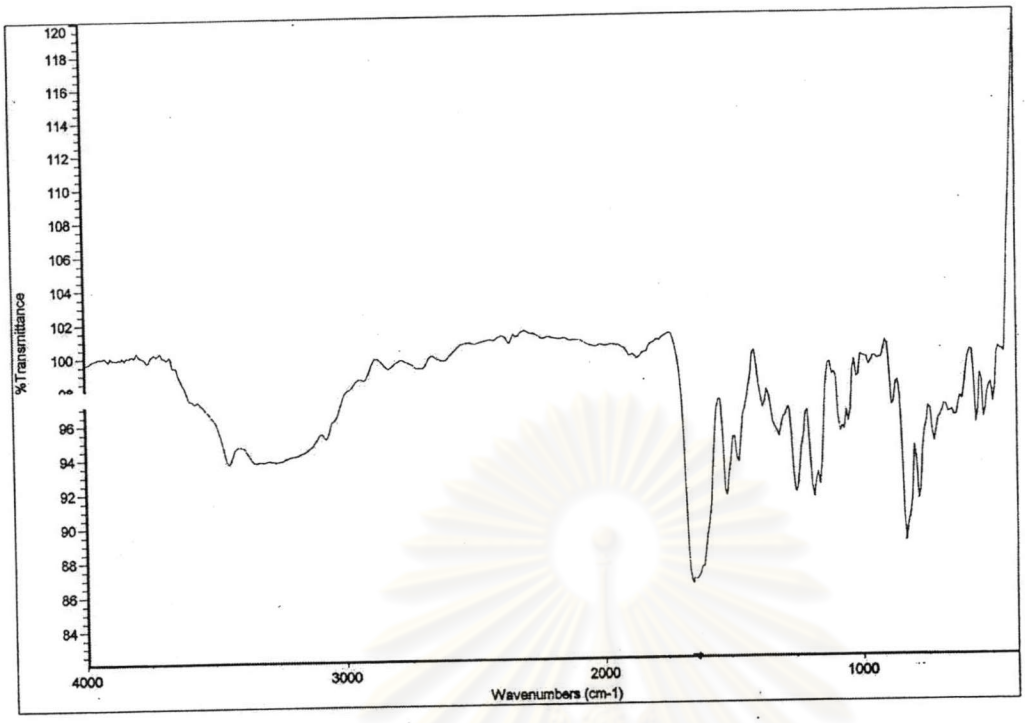


Figure 3.2 IR spectrum of compound **1**

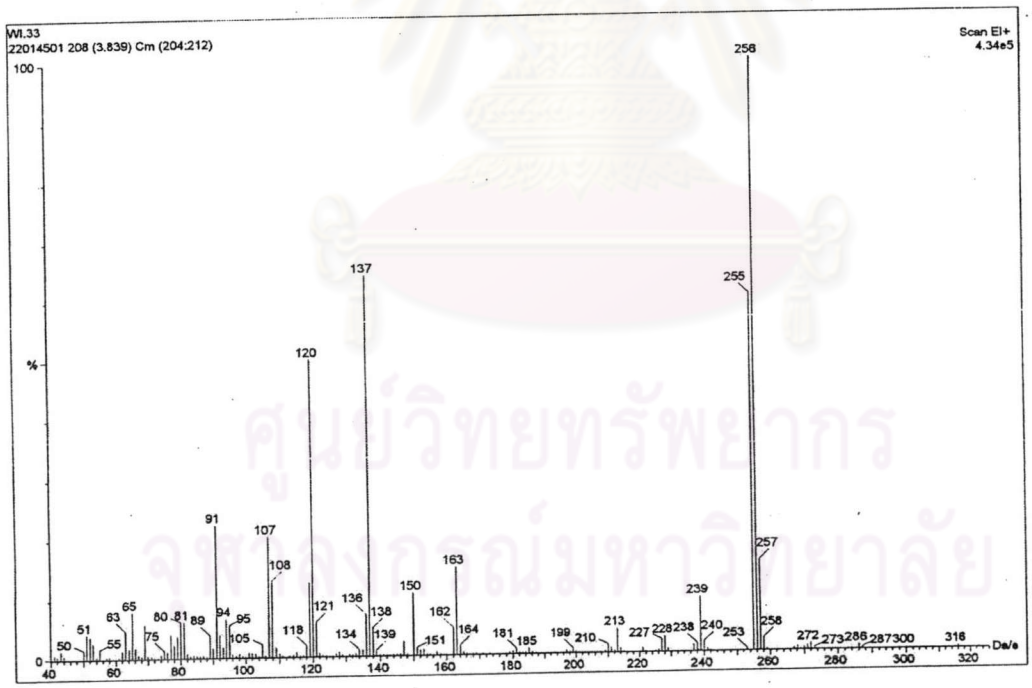


Figure 3.3 Mass spectrum of compound **1**

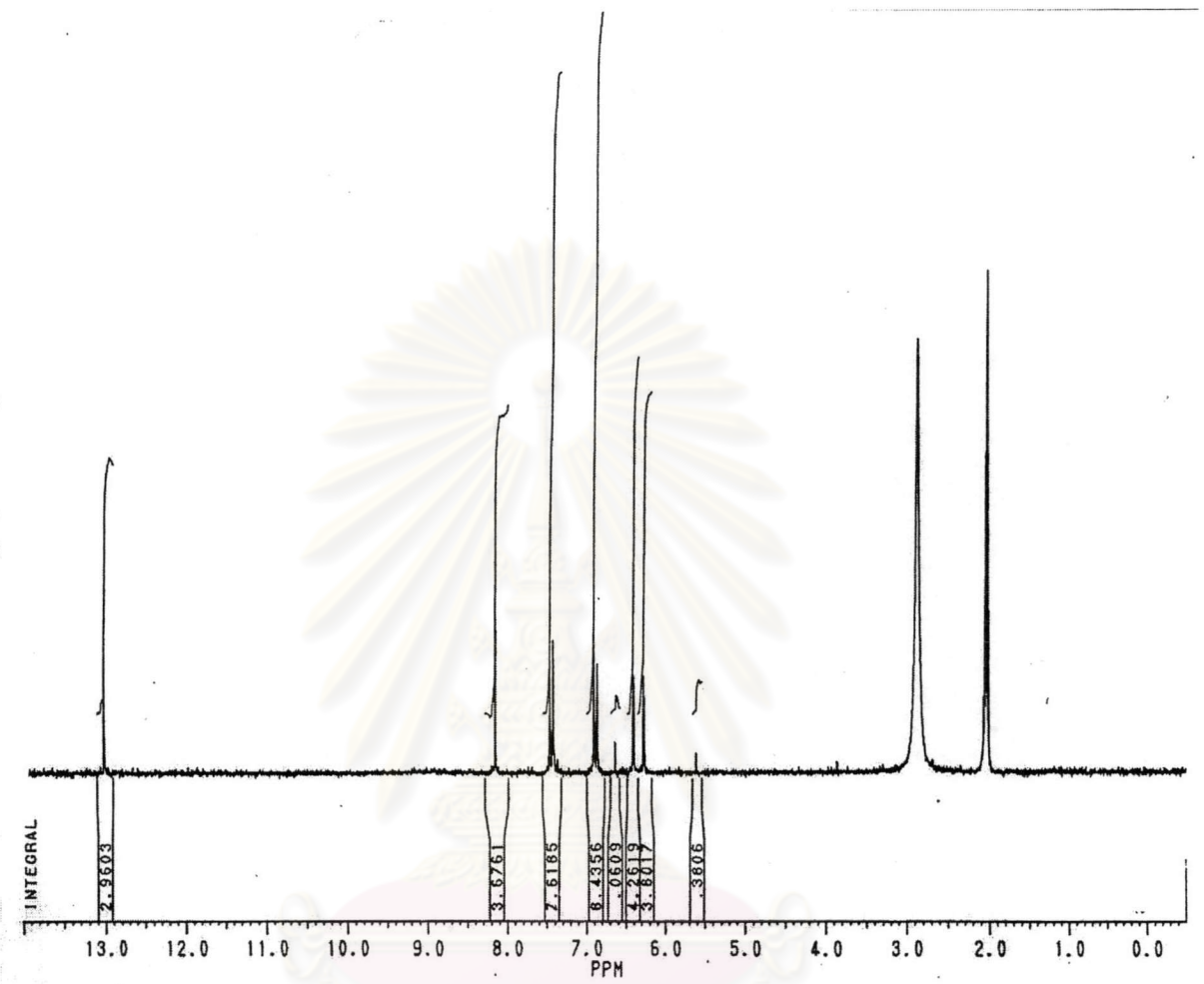


Figure 3.4 ^{1}H-NMR spectrum of compound **1**

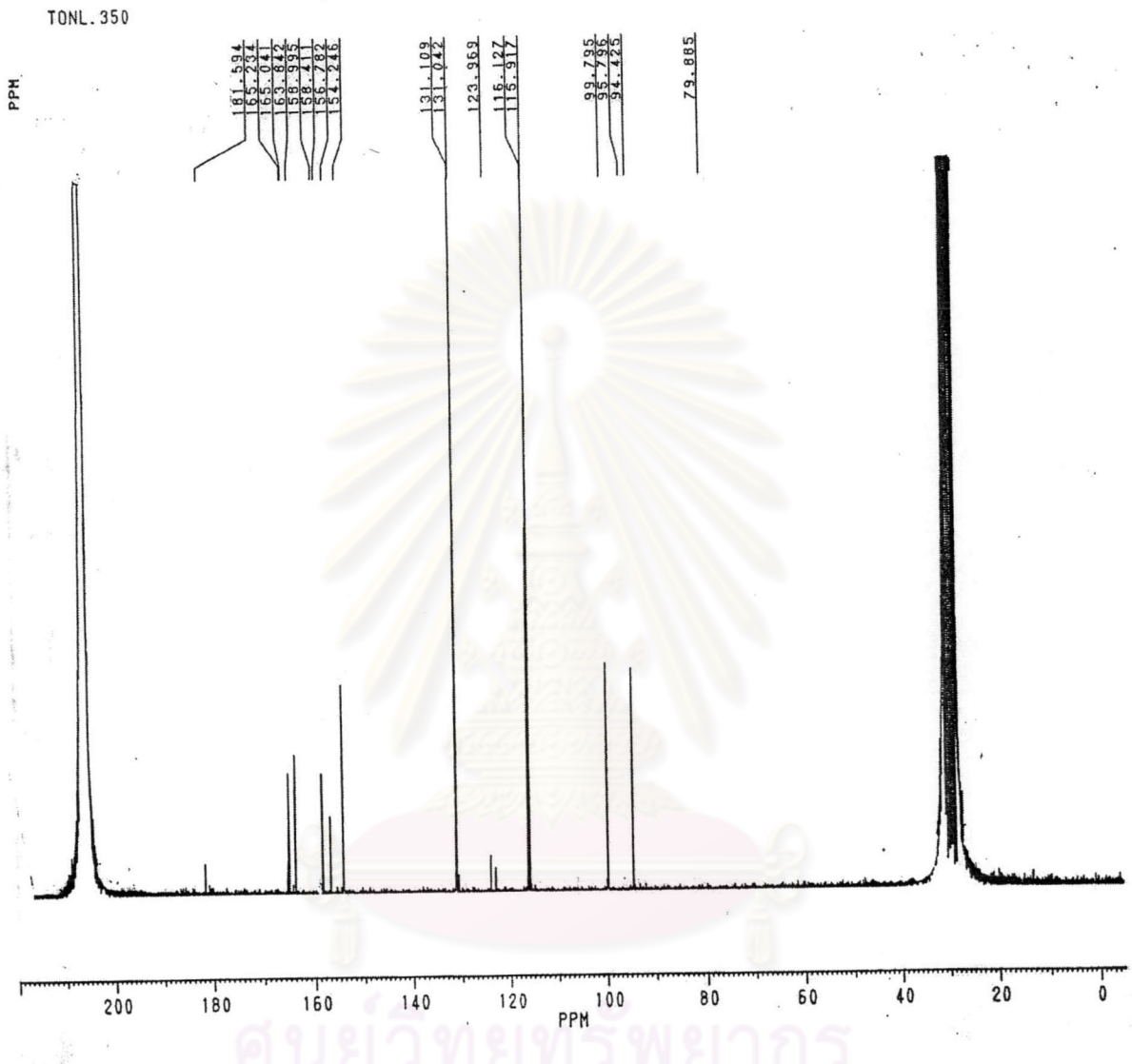


Figure 3.5 ^{13}C-NMR spectrum of compound **1**

3.2.2 Compound 2

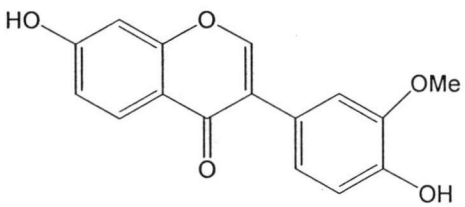
Compound **2** was isolated as yellow-green needles (1.9 g) from open column chromatography of ethyl acetate crude extract. Its melting point was 205-208 °C and R_f 0.48 (SiO_2, EtOAc-CH_2Cl_2, 6:4). It was soluble in methanol and acetone but not in hexane, dichloromethane and ethyl acetate.

The IR spectrum (**Figure 3.6**) clearly confirmed the presence of hydroxyl group at ν_{max} 3100-3400 cm^{-1} due to -OH stretching vibration, carbonyl group at ν_{max} 1634 cm^{-1} due to C=O stretching vibration and benzene moiety at ν_{max} 1567, 1511 and 1490 cm^{-1}.

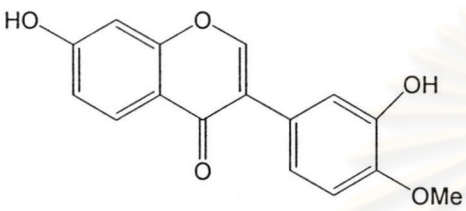
The mass spectrum (**Figure 3.7**) of compound **2** showed a molecular ion peak at m/z 284 [M^+] along with fragment ion peaks at m/z 269, 241, 213, 162, and 137 in the electron impact mass spectrum (EIMS). From EIMS, ^1H and ^{13}C NMR data, compound **2** was determined to be $C_{16}H_{12}O_5$, indicating 11 degrees of unsaturation.

The ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.8**) displayed signals of six aromatic protons at δ 6.89 (1H, d, J = 2.1 Hz), 6.99 (1H, dd, J = 2.1, 8.1 Hz), 7.05 (1H, dd, J = 2.1, 8.2 Hz), 7.15 (1H, d, J = 2.1 Hz) and 8.05 (1H, d, J = 8.2 Hz). The proton doublets, meta-coupled, showed a coupling constant of 2.1 Hz (H-8 and H-2' respectively). The coupling constant of 8.2 Hz showed proton doublet of ortho coupled (H-5, H-5'). Two one proton doublet of doublet (J = 2.1, 8.2 Hz) meta and para coupled to each other were assigned to H-6 and H-6'. One proton attached to the sp^2 carbon at δ 8.15 (1H, s) and a methoxy group at δ 3.86 ppm. From this data, compound **2** should be an isoflavone or a flavone derivative.

The ^{13}C-NMR spectrum (**Figure 3.9**) of this compound indicated sixteen carbons of fifteen skeletons and one substituent group (characteristic of flavonoid). There were seven methine carbons [δ 128.4 (C-5), 115.6 (C-6), 103.1(C-8), 153.4 (C-2), 112.0 (C-2'), 116.8 (C-5') and 121.0 (C-6')], eight quaternary carbons [δ 163.1 (C-7), 158.6 (C-9), 118.5 (C-10), 175.5 (C-4), 126.2 (C-3), 125.0 (C-1'), 148.1 (C-3') and 146.9 (C-4')] and one methoxy group (δ 56.2). From the evidence of the ^1H, ^{13}C-NMR and mass spectral data, compound **2** should be 3'-methoxydiadzein or 3'-hydroxyformononetin (calycosin).

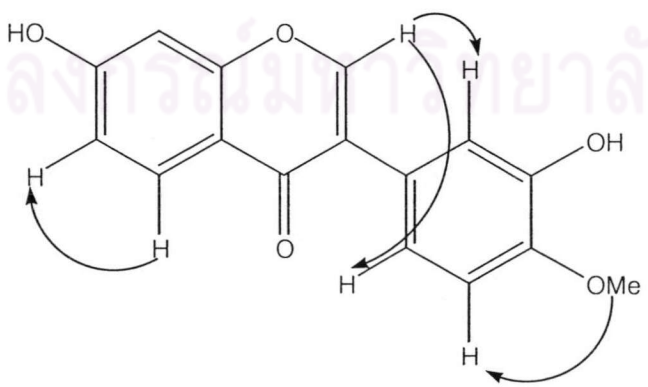


3'-Methoxydaidzein



3'-hydroxyformononetin
or calycosin

The NOE DIFF experiments were used to obtain additional information. Irradiation of the proton signal at δ 8.15 ppm caused enhancement of the proton signal at δ 7.15 ppm (H-2') and 7.05 ppm (H-6'); irradiation of the proton signal at δ 8.05 ppm only caused enhancement of the proton signal at δ 6.99 ppm (H-6) and irradiation in methoxy protons at δ 3.86 ppm caused enhancement of the proton signal at δ 6.97 ppm (H-5') (**Figure 3.10-3.12**). The NOE DIFF correlation of compound **2** was shown below. The proton and carbon assignments of compound **2** were displayed in **Table 3.6**. The structure of this compound was also confirmed by comparison with reported data of calycosin (**Table 3.7**).



The NOE DIFF correlation of compound **2**

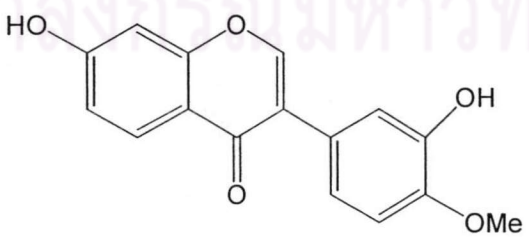
Table 3.6 ^{1}H and ^{13}C spectral data of compound **2**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	153.4	8.15	1H, s
3	126.2		
4	175.5		
5	128.4	8.05	1H, d, J = 8.2
6	115.6	6.99	1H, dd, J = 2.1, 8.1 Hz
7	163.1		
8	103.1	6.89	1H, d, J = 2.1 Hz
9	158.6		
10	118.5		
1'	125.0		
2'	112.0	7.15	1H, d, J = 2.1 Hz
3'	148.1		
4'	146.9		
5'	116.8	6.97	1H, d, J = 8.2 Hz
6'	121.0	7.05	1H, dd, J = 2.1, 8.2 Hz
-OMe	56.2	3.86	3H, s

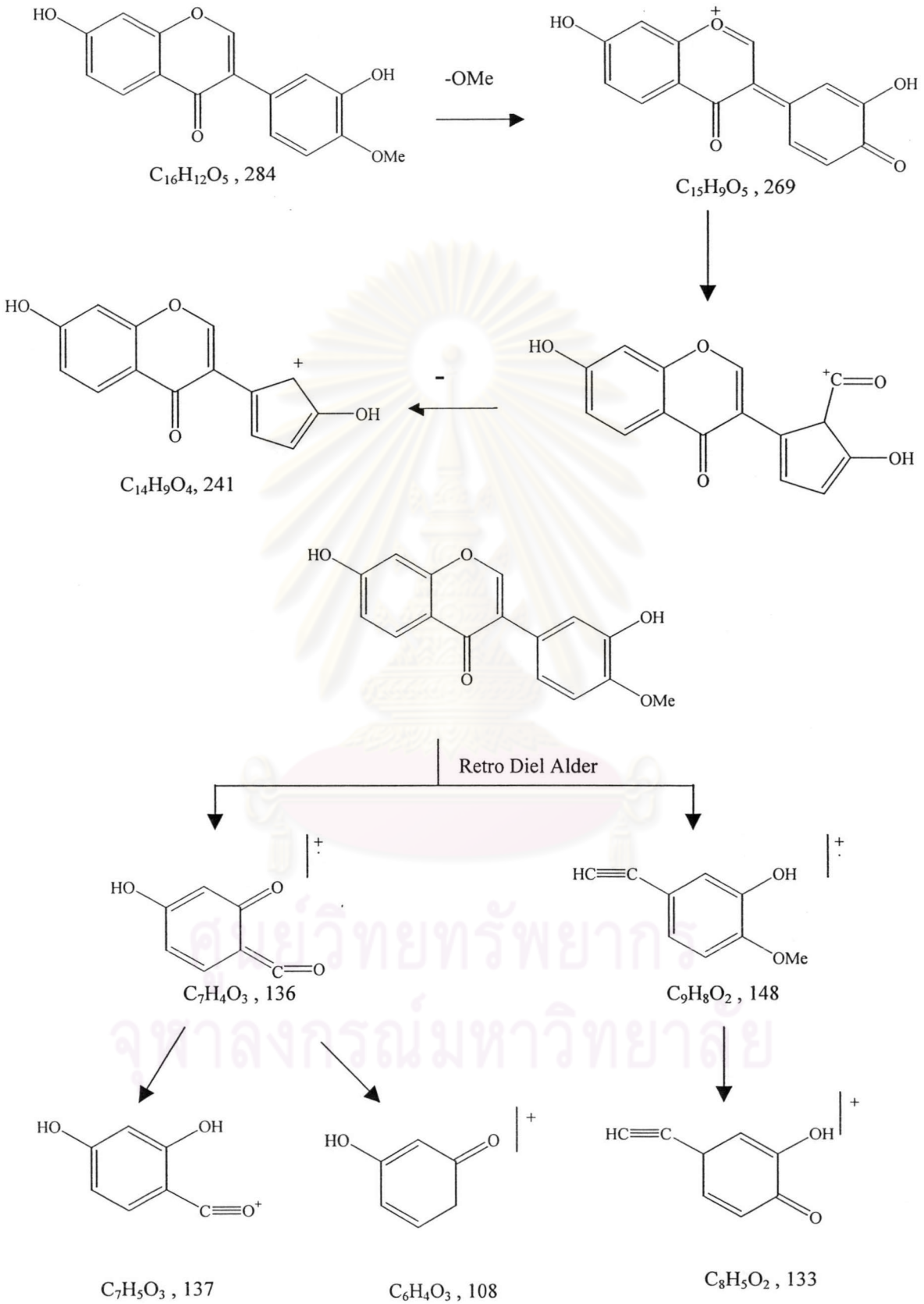
Table 3.7 ^{13}C NMR data of compound **2** compared with 3'-Methoxydaidzein.[22]

Position	Compound **2**	3'-Methoxydaidzein
2	153.4	152.8
3	126.2	124.7
4	175.5	174.6
5	128.4	116.7
6	115.6	115.1
7	163.1	162.5
8	103.1	102.1
9	158.6	157.4
10	118.5	116.7
1'	125.0	123.5
2'	112.0	111.9
3'	148.1	147.5
4'	146.9	146.1
5'	116.8	116.5
6'	121.0	119.7
-OMe	56.2	55.6

This spectroscopic observation indicated that compound **2** was 3'-hydroxyformononetin or calycosin.



Structure of compound **2** (calycosin)



Scheme 3.2 Possible mass fragmentation patterns of compound **2**

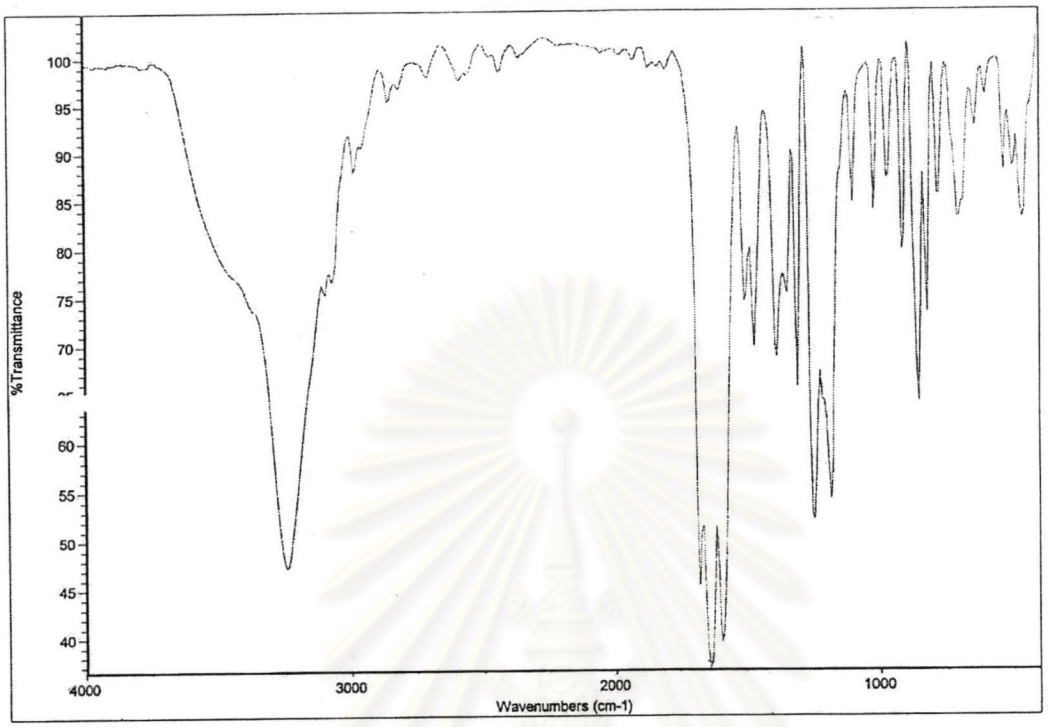


Figure 3.6 IR spectrum of compound **2**

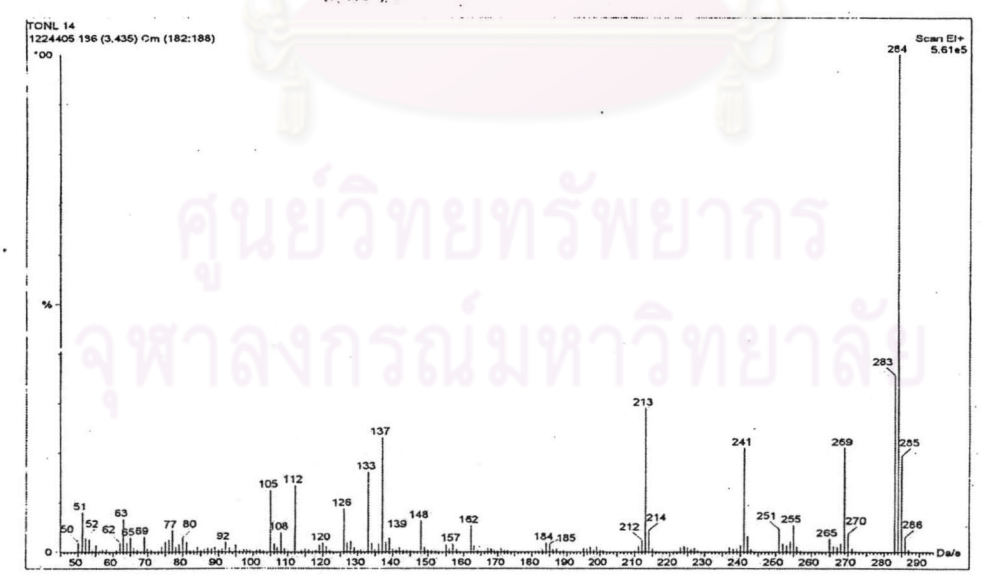


Figure 3.7 Mass spectrum of compound **2**

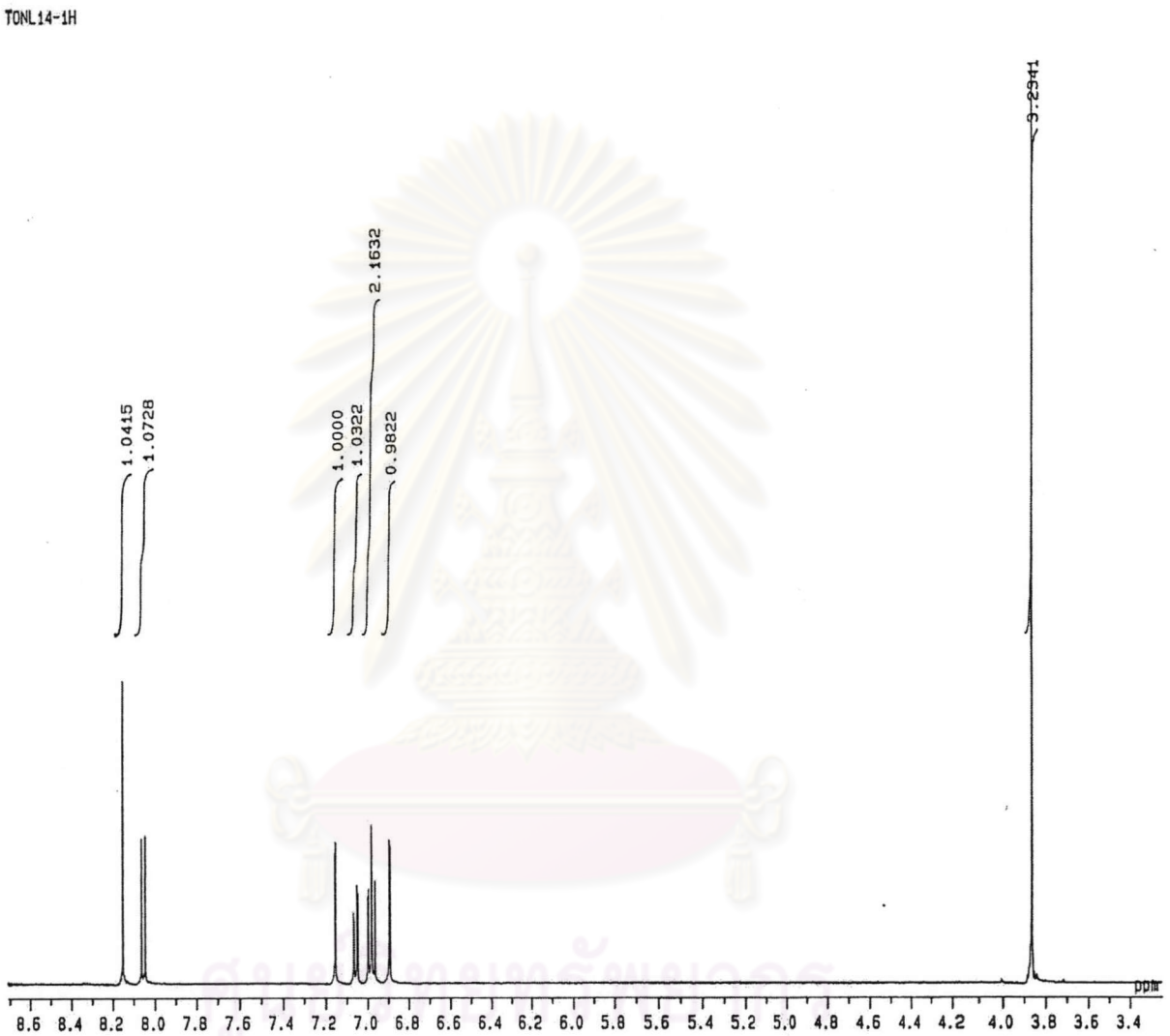


Figure 3.8 ^{1}H-NMR spectrum of compound **2**

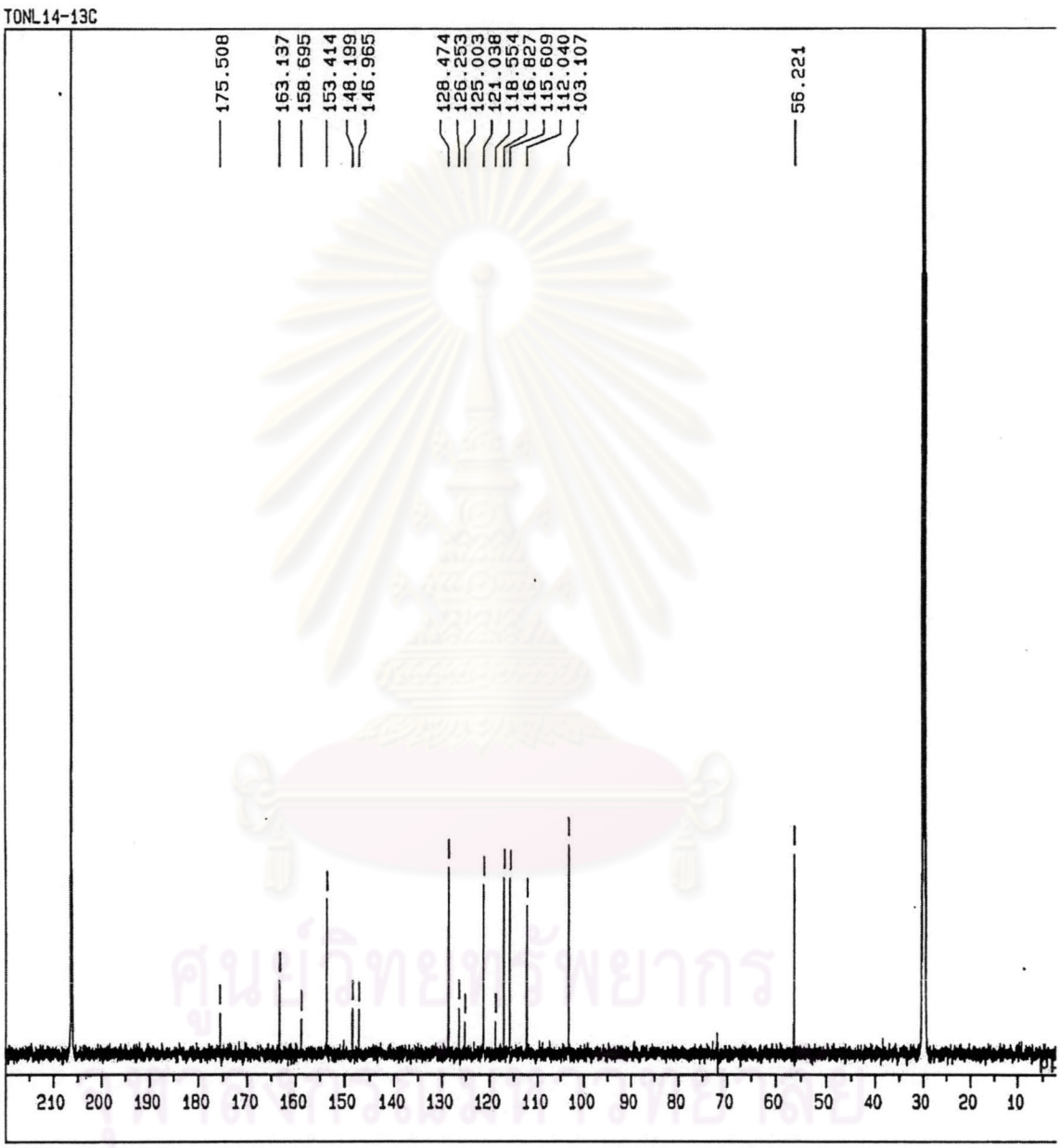


Figure 3.9 ^{13}C-NMR spectrum of compound **2**

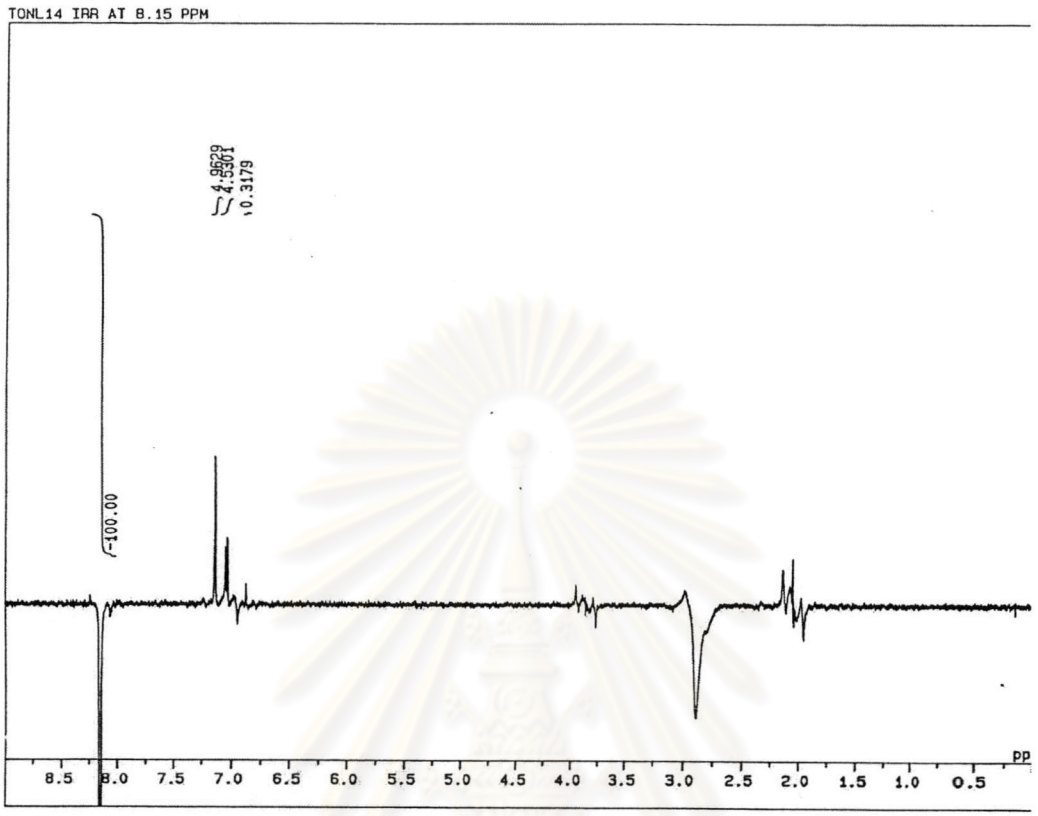


Figure 3.10 NOE DIFF of compound **2** (irradiated at 8.15 ppm)

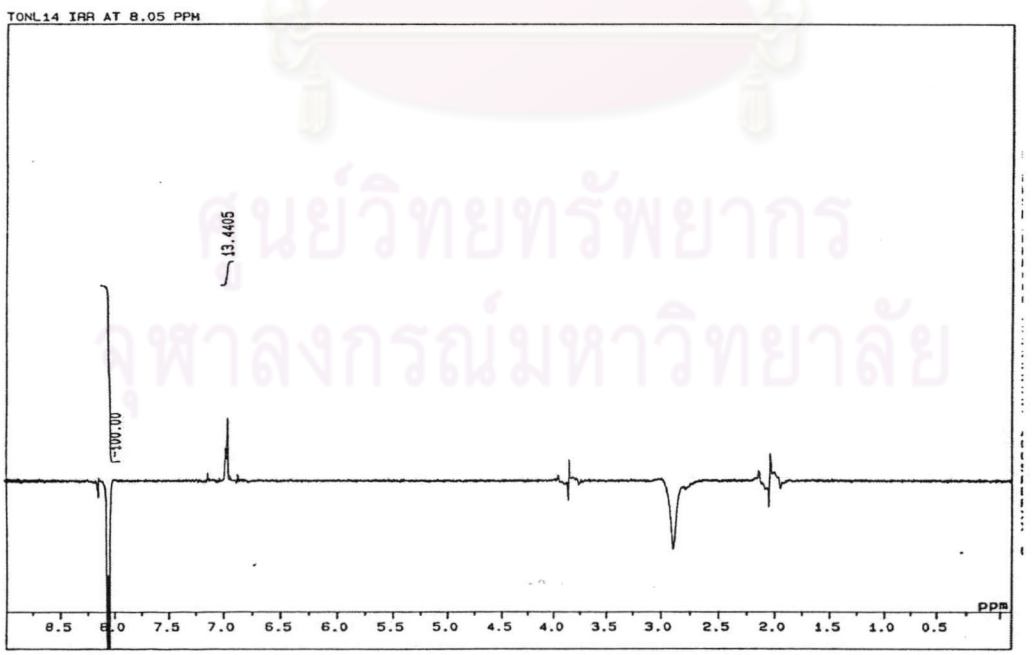


Figure 3.11 NOE DIFF of compound **2** (irradiated at 8.05 ppm)

3.2.3 Compound 3

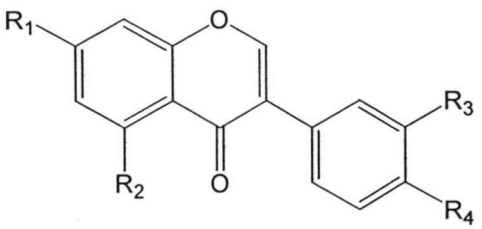
Compound **3** was yielded as white needles (1.30g) from open column chromatography of ethyl acetate crude extract and further purified by recrystallization in acetone. Its melting point was 236-238 °C and R_f was 0.62 (SiO_2, EtOAc-CH_2Cl_2, 6:4). It was soluble in acetone and slightly soluble in methanol but not in ethyl acetate, dichloromethane and hexane. Molecular formula was determined to be $C_{16}H_{12}O_6$ on the basis of ^1H, ^{13}C-NMR and EIMS data.

The mass spectrum (**Figure 3.13**) of this compound showed the molecular ion peak at m/z 300 [M^+] along with fragment ion peaks at m/z 285, 271, 257, 229, 153, 133 and 120.

The IR spectrum (**Figure 3.14**) indicated the presence of free hydroxyl groups at ν_{max} 3318 cm^{-1}, chelated hydroxyl group at ν_{max} 3446 cm^{-1}, a carbonyl group which conjugated with aromatic rings and also hydrogen-bonded with hydroxyl group at ν_{max} 1659 cm^{-1} and benzene ring at ν_{max} 1623, 1516 and 1470 cm^{-1}.

The ^1H-NMR (acetone-d_6, 500MHz) spectrum of compound **3** (**Figure 3.15**) revealed five aromatic proton signals at δ 7.13 (H-2', d, J = 2.1 Hz), 7.04 (H-6', dd, J = 2.1, 8.2 Hz), 6.99 (H-5', d, J = 8.2 Hz), 6.41 (H-6, d, J = 2.1 Hz) and 6.27 (H-8, d, J = 2.1 Hz), a proton attached to the sp^2 carbon at 8.18 (1H, s), a singlet signal at δ 13.03 (proton signal disclosed low field, this hydroxy formed hydrogen bonds with the carbonyl group) and the presence of the methoxy group was indicated by a singlet signal at δ 3.87.

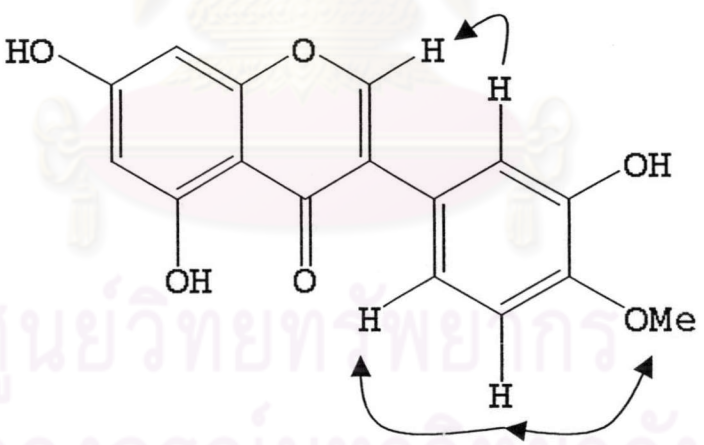
The ^{13}C-NMR spectrum (**Figure 3.16**) of this compound indicated sixteen carbons of fifteen skeletons and one substituent group (characteristic of flavonoid), compound **3** had one methoxy carbon at δ 56.2 (-OMe), six methine carbons at δ 94.4 (C-8), 99.8 (C-6), 112.1 (C-2'), 116.8 (C-5'), 121.1 (C-6') and 154.5 (C-2) and nine quaternary carbons at δ 163.8 (C-5), 165.0 (C-7), 158.9 (C-9), 106.1 (C-10), 124.8 (C-3), 123.8 (C-1'), 147.1 (C-3'), 148.4 (C-4') and 181.5 (C=O). All of the above data suggested the possibility of a flavonoid skeleton of isoflavone type. Therefore, the data and comparison with ^1H, ^{13}C-NMR of known isoflavonoid suggested that molecule of this compound must have the basic skeleton as shown below:



Substituents :

- OH (3)
- OMe (1)

The position of substitution of the methoxy group was confirmed by the NOE difference technique; irradiation of the methoxy proton signal at δ 3.87 only caused enhancement of the proton signal at δ 6.99 (H-5'); irradiation of the proton signal at δ 6.99 caused enhancement of the proton signal at δ 7.04 (H-6') and methoxy protons signal at δ 3.87 and irradiation of the proton signal at δ 7.14 only caused enhancement of the proton signal at δ 8.18 (H-2) (**Figure 3.17-3.19**). The NOE DIFF correlation of compound **3** was displayed below. The assignment of protons and carbons of this compound was also indicated in **Table 3.8**.

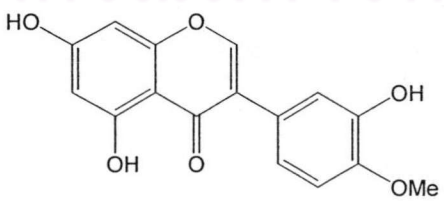


The NOE DIFF correlation of compound **3**

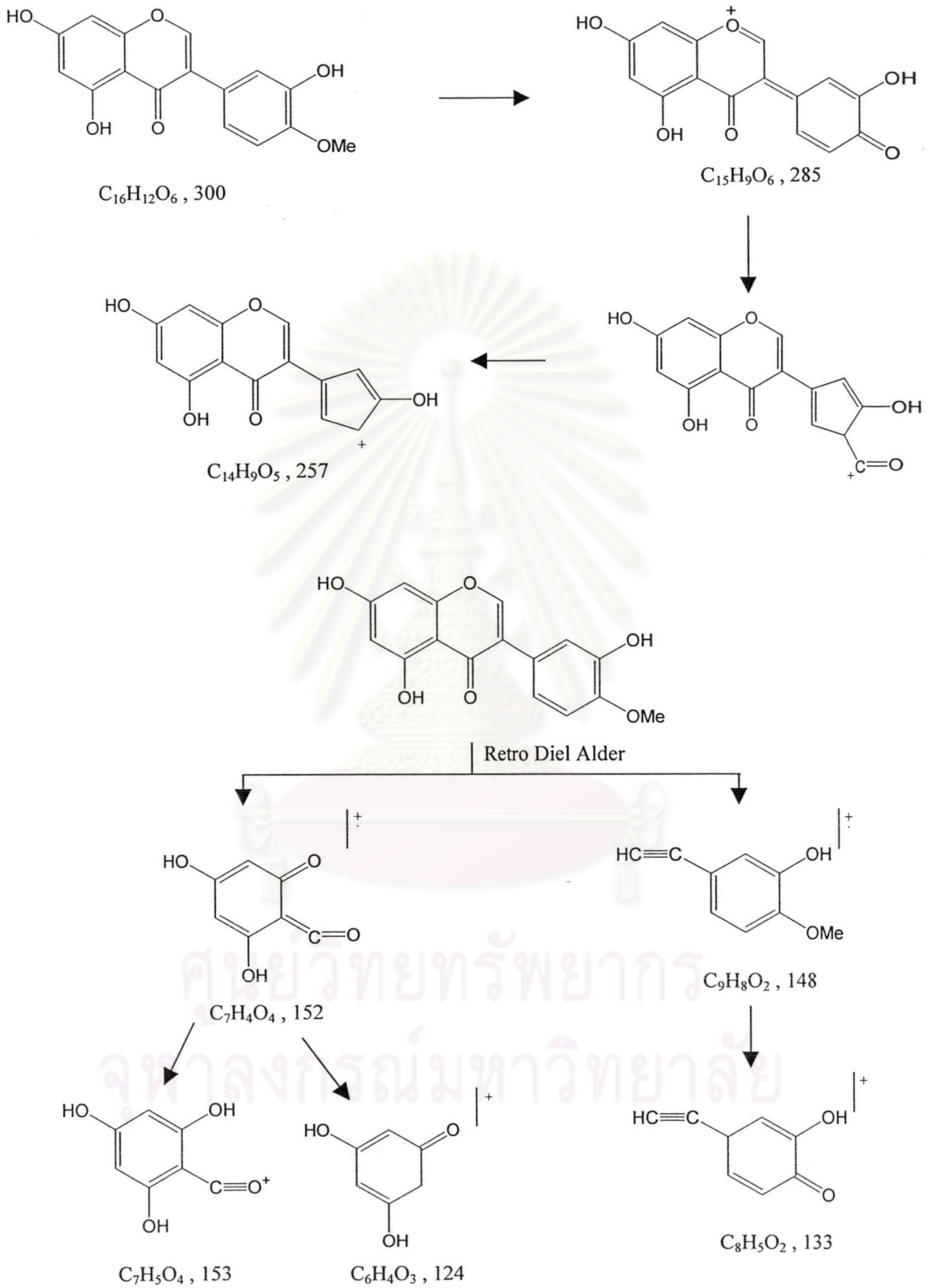
Table 3.8 ^{1}H and ^{13}C spectral data of compound **3**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	154.2	8.18	1H, s
3	124.8		
4	181.5		
5	163.8		
6	99.8	6.41	1H, d, J = 2.1 Hz
7	165.0		
8	94.4	6.27	1H, d, J = 2.1 Hz
9	158.9		
10	106.1		
1'	123.8		
2'	112.1	7.13	1H, d, J = 2.1 Hz
3'	147.1		
4'	148.4		
5'	116.8	6.99	1H, d, J = 8.2 Hz
6'	121.1	7.04	1H, dd, J = 2.1, 8.2 Hz
-OH	-	13.03	1H, s
-OMe	56.2	3.87	3H, s

On the basis of all the spectroscopic data, the structure of compound **3** was established as 5,7,3'-trihydroxy-4'-methoxyisoflavone or pratensein.[23]



Structure of compound **3** (pratensein)



Scheme 3.3 Possible mass fragmentation patterns of compound **3**

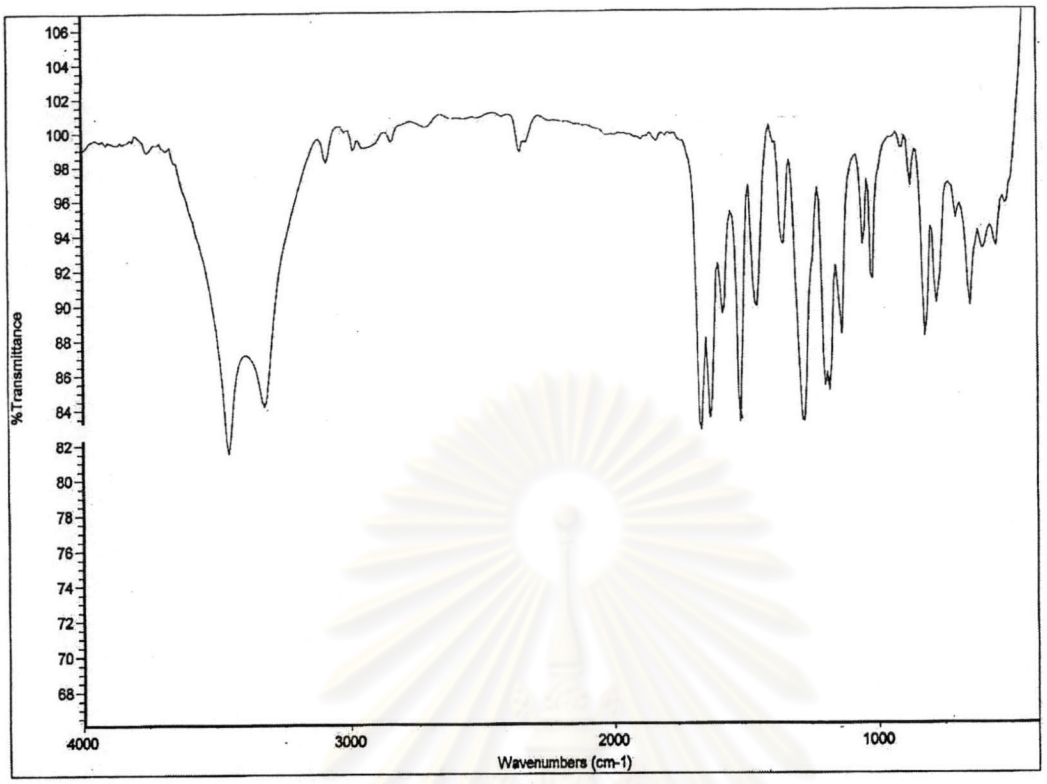


Figure 3.13 IR spectrum of compound **3**

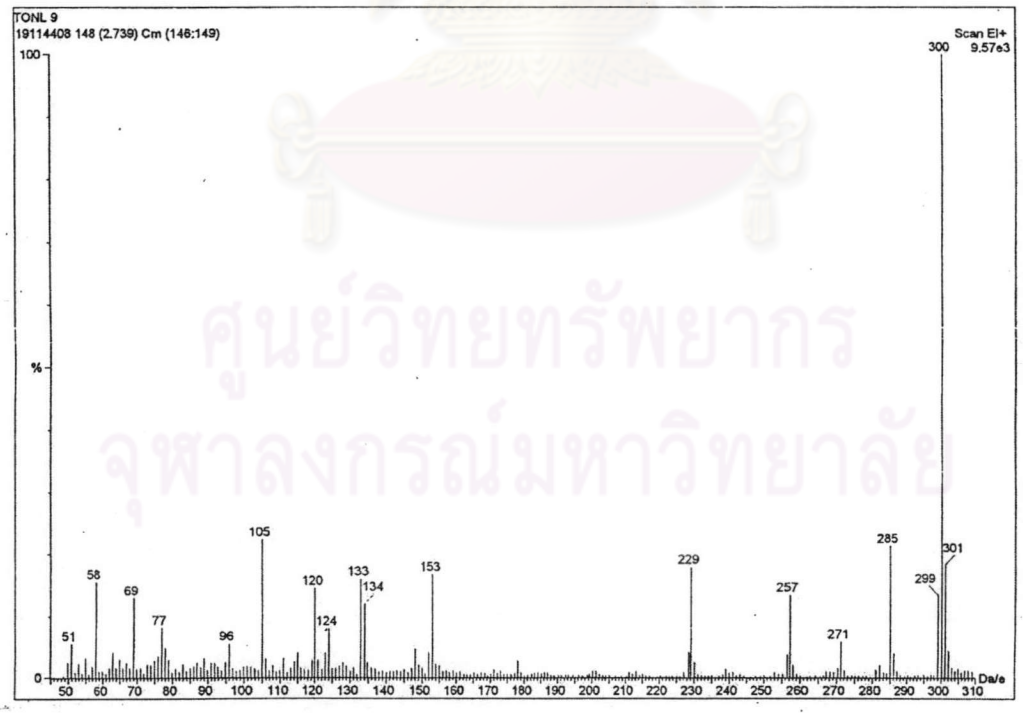


Figure 3.14 Mass spectrum of compound **3**

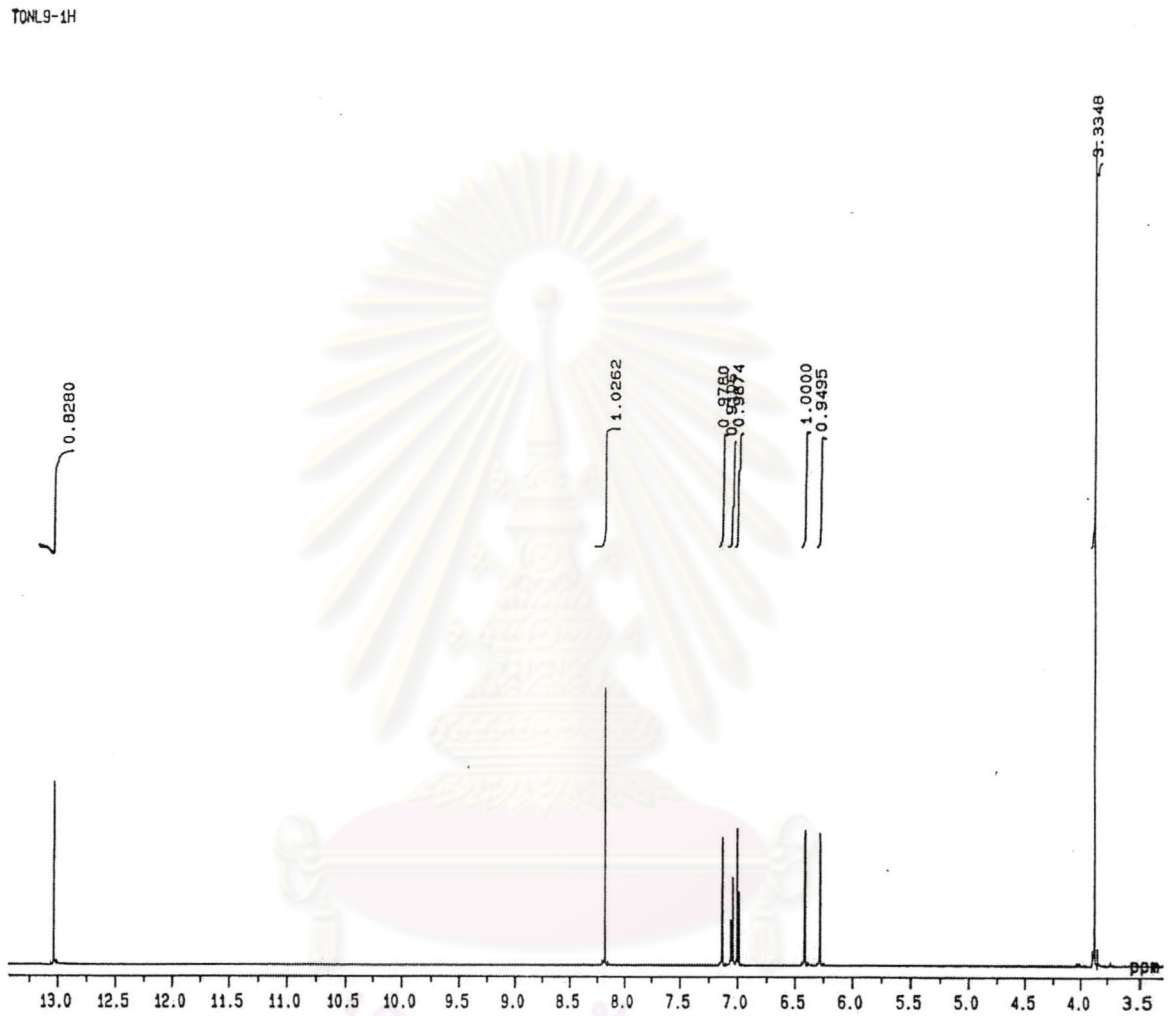


Figure 3.15 ^{1}H-NMR spectrum of compound **3**

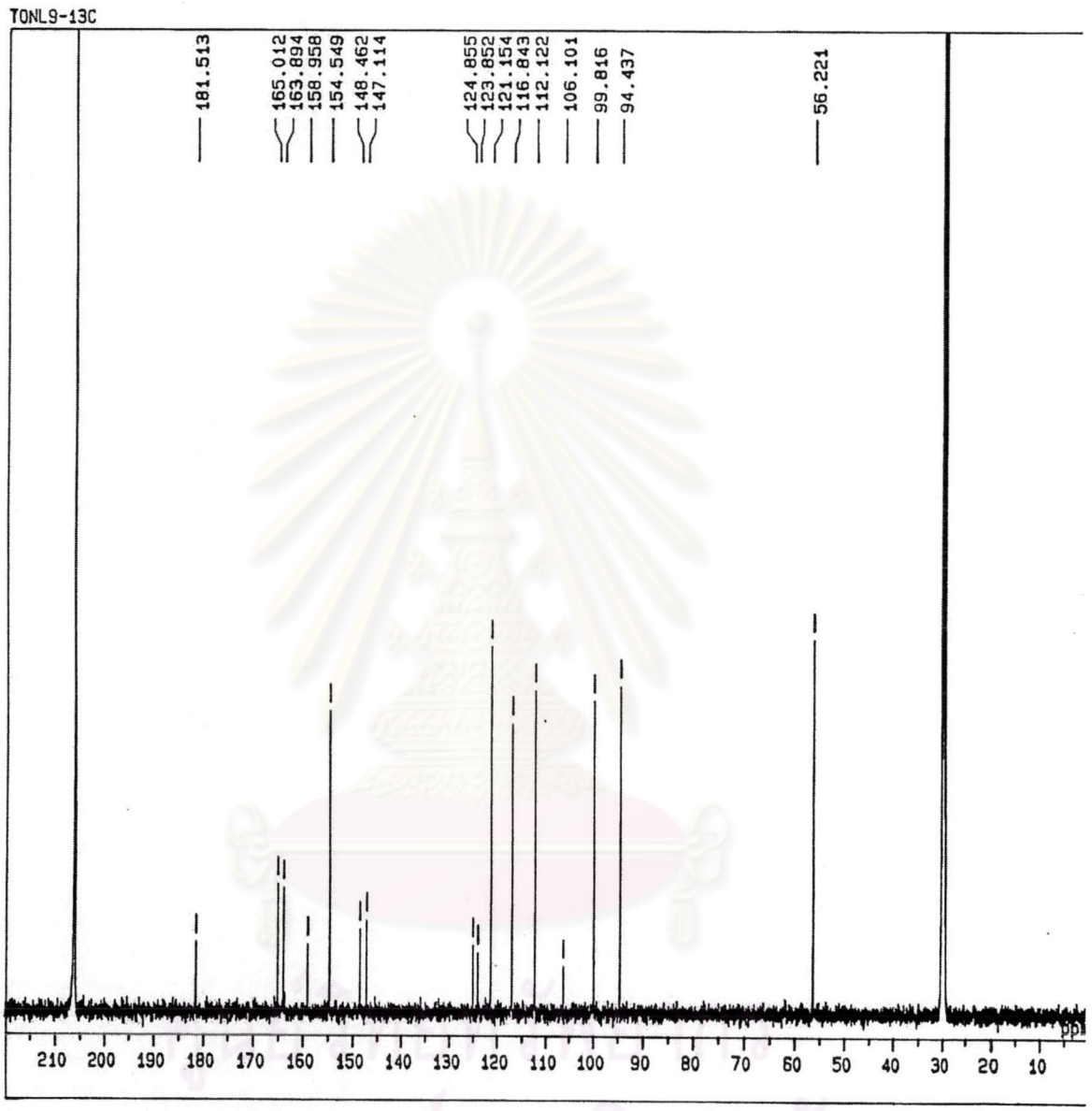


Figure 3.16 ^{13}C-NMR spectrum of compound **3**

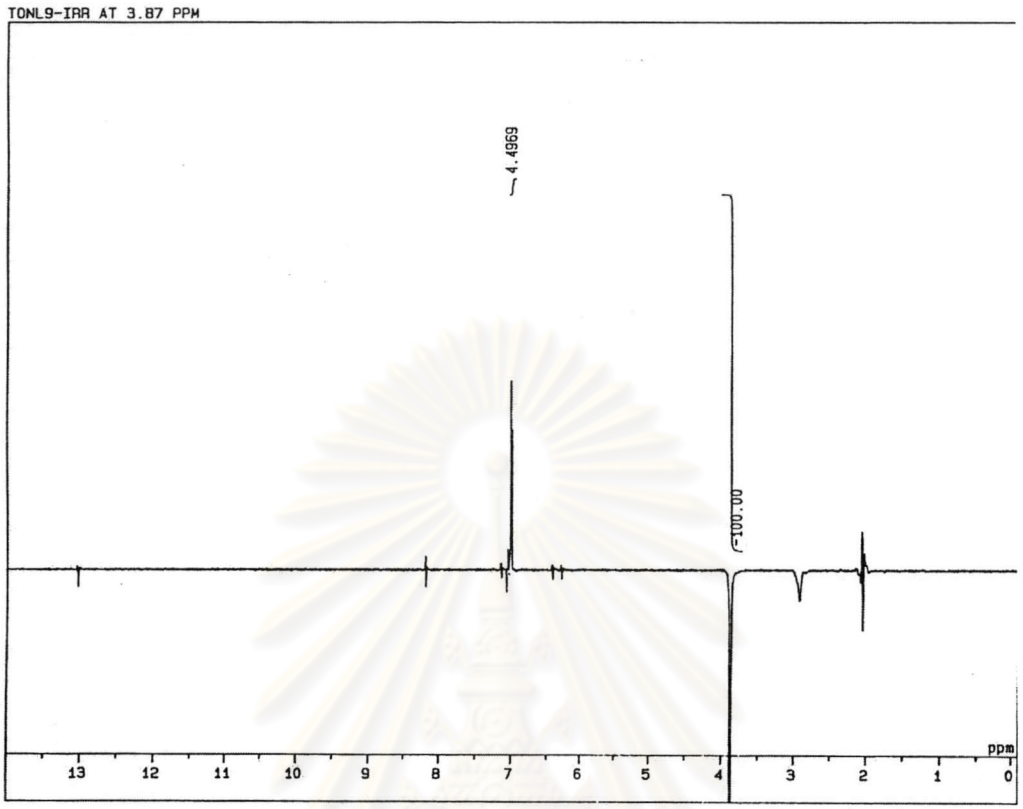


Figure 3.17 NOE DIFF of compound **3** (irradiated at 3.87 ppm)

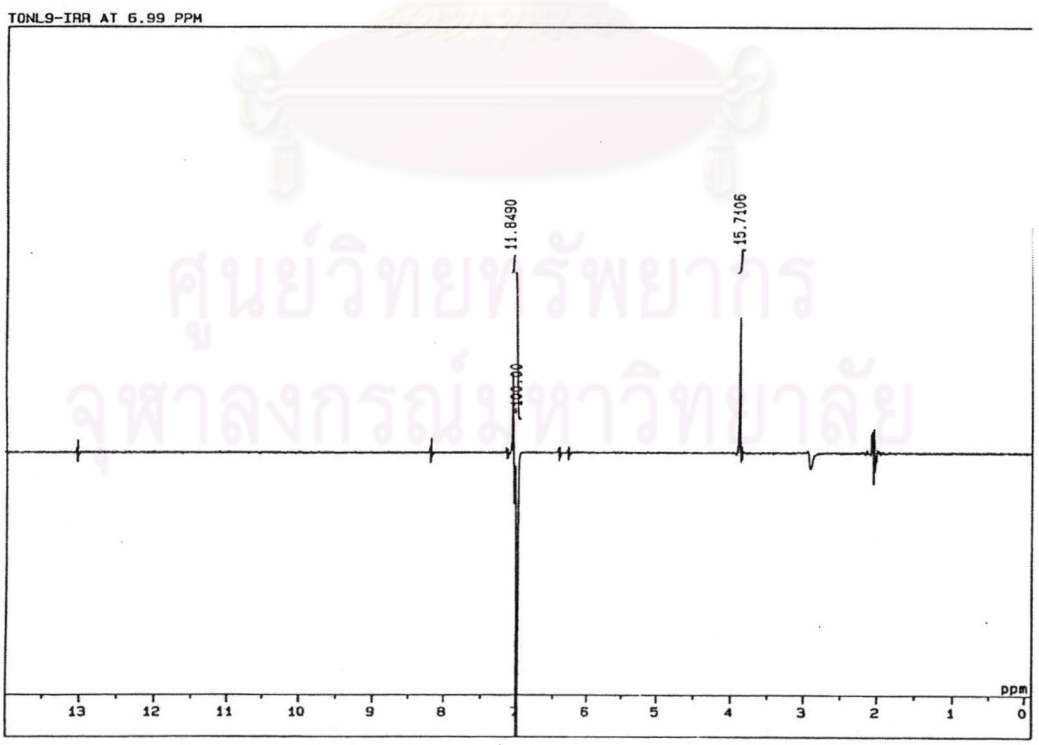


Figure 3.18 NOE DIFF of compound **3** (irradiated at 6.99 ppm)

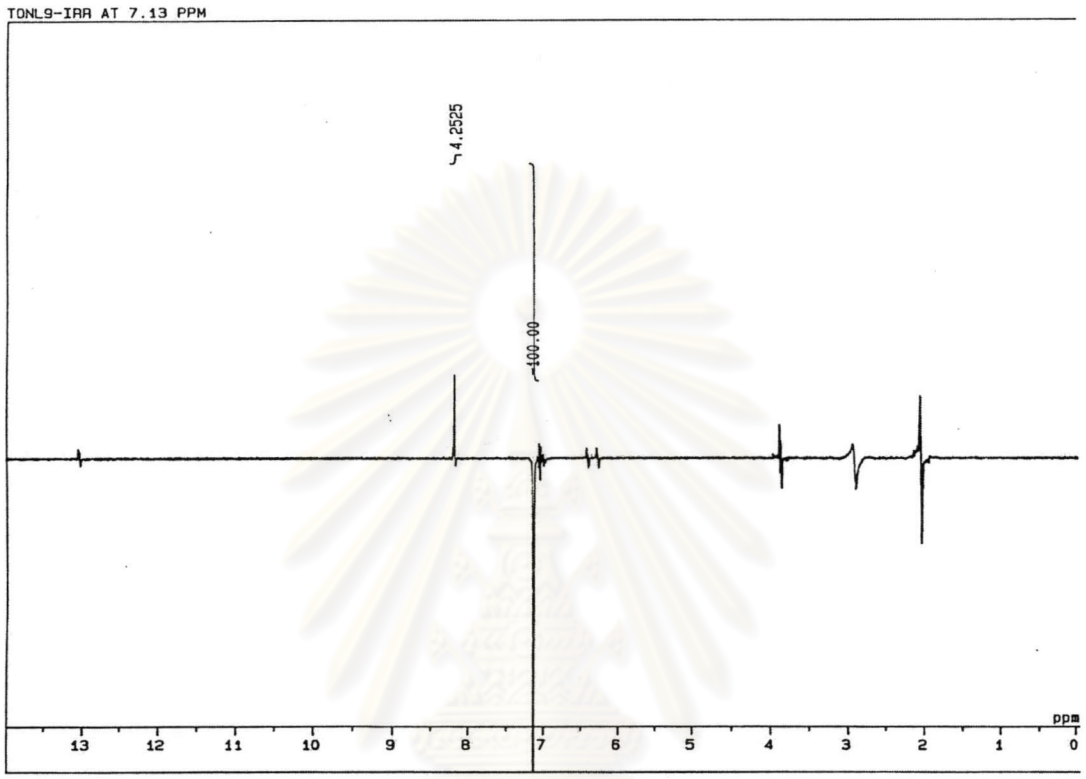


Figure 3.19 NOE DIFF of compound **3** (irradiated at 7.13 ppm)

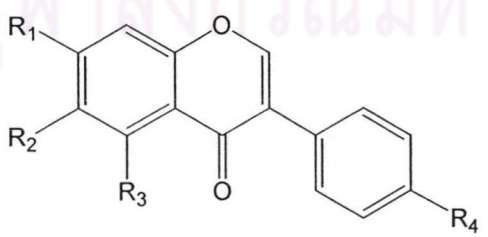
3.2.4 Compound 4

Compound **4** appeared as white needles crystals with melting point 204-207 °C after recolumn chromatography and recrystallization in acetone and R_f value 0.44 (SiO_2, EtOAc-CH_2Cl_2,2:8). The formula of compound **4** was established as $C_{16}H_{12}O_6$ by Electron Impact Mass Spectroscopy (EIMS) (**Figure 3.20**) ([M^+] m/z 300), with significant or intense fragment ions at m/z 285, 257 and 150.

The IR spectrum **(Figure 3.21)** showed strong absorption at ν_{max} 3477, 3272 and 1639 cm^{-1} which indicated the presence of chelated hydroxyl group, free hydroxyl group and a carbonyl group which conjugated with a double bond, respectively and benzene moiety at ν_{max} 1572, 1490 and 1465 cm^{-1}.

From the ^{1}H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.22**) revealed five aromatic proton signals for p-disubstituted aromatic protons at δ 7.47 (2H, d, J = 8.6 Hz) and δ 6.91 (2H, d, J = 8.6 Hz), a one proton singlet at δ 6.50 (1H, s), one methyl protons of methoxy group at δ 3.86 and a singlet signal at δ 8.18 which suggested that this compound might be an isoflavone type.

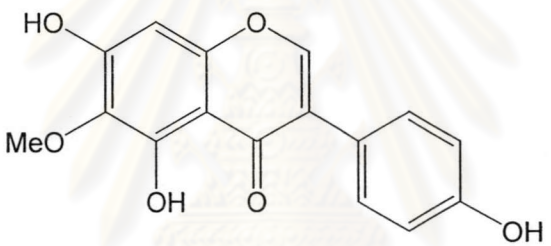
The ^{13}C-NMR spectrum (**Figure 3.23**) indicated sixteen carbons of fifteen skeletons and one methoxy group (flavonoid characteristic); methyl carbon of methoxy group at δ 60.6 (-OMe). This compound contained nine quaternary carbons at δ 123 (C-3), 182.0 (C-4, C=O), 154.4 (C-5), 132.1 (C-6), 154.5 (C-7), 154.2 (C-9), 106.4 (C-10), 122.9 (C-1') and 157.7 (C-4'); six methine carbons at δ 158.3 (C-2), 94.3 (C-8), 131.1 (2C, C-2', C-6') and 122.9 (2C, C-3', C-5'). Therefore, all of the above spectroscopic data was proposed as isoflavone with three substituted on ring A and one substituted on ring B, the molecule of this compound must have the basic skeleton showed below:



Substituents ;
- OH (3)
- OMe (1)

The position of a substitution (methoxy) group was confirmed by the NOE difference technique. Irradiattion of the proton signal at δ 8.18 caused an enhancement of the proton signal at δ 7.47 (H-2') and irradiation of methoxy proton signals at δ 3.86 had no effect on any aromatic proton (**Figures 3.24 – 3.25**). The assignment of protons and carbons of compound **4** was displayed in **Table 3.9**.

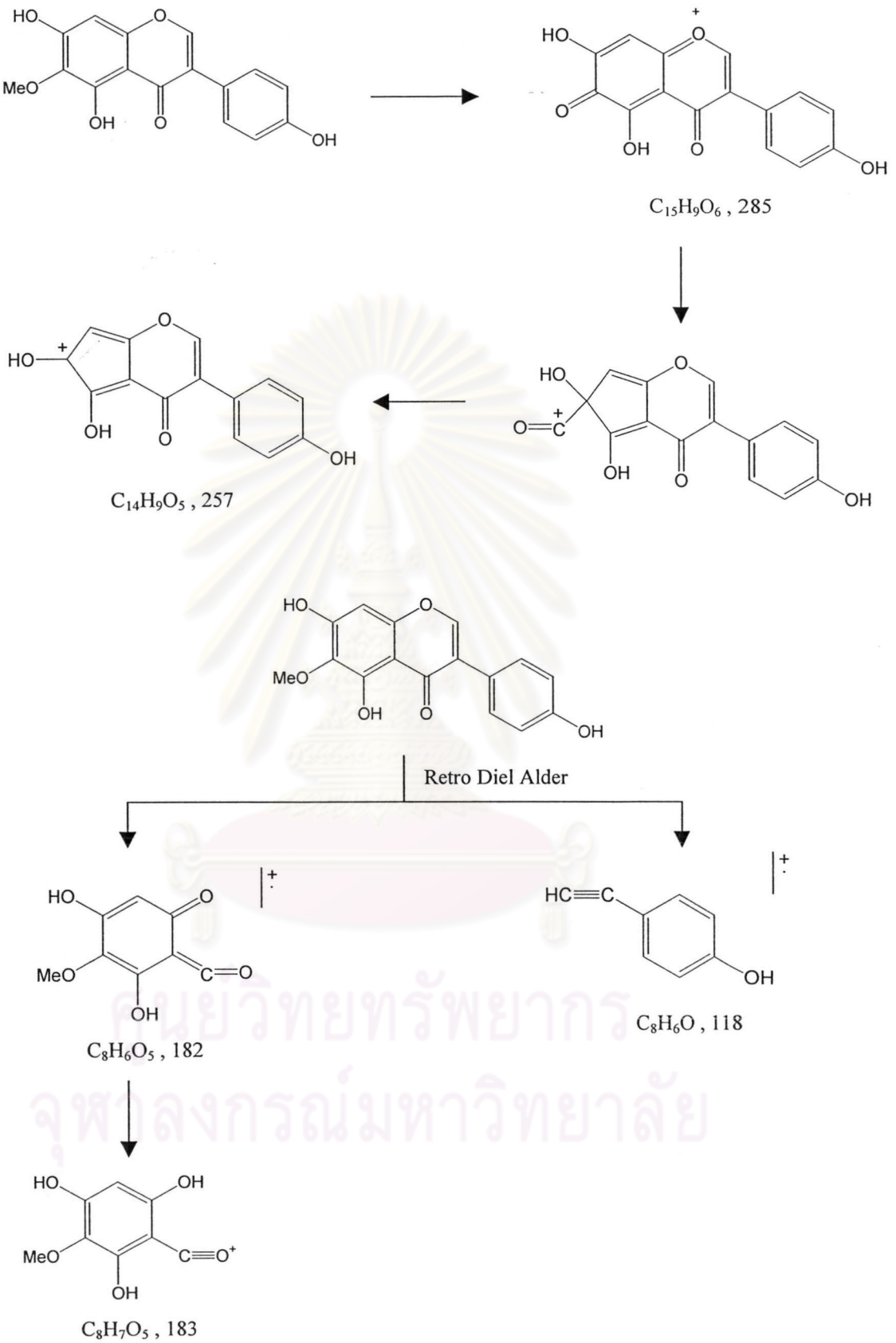
Based on the above spectral characteristic, and comparison of the ^{1}H-NMR and mass spectrum data of compound **4** with published data of tectorigenin, the structure of compound **4** was elucidated as tectorigenin.[23]



The structure of compound **4** (tectorigenin)

Table 3.9 ^{1}H and ^{13}C spectral data of compound **4**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	158.3	8.18	1H, s
3	123.4		
4	182.0		
5	154.4		
6	132.1		
7	154.5		
8	94.3	6.50	1H, s
9	154.2		
10	106.4		
1'	122.9		
2'	131.1	7.47	1H, d, J = 8.6 Hz
3'	122.9	6.91	2H, d, J = 8.6 Hz
4'	157.7		
5'	122.9	6.91	1H, d, J = 8.6 Hz
6'	131.1	7.47	1H, d, J = 8.2 Hz
-OMe	60.6	3.86	3H, s



Scheme 3.4 Possible mass fragmentation patterns of compound **4**

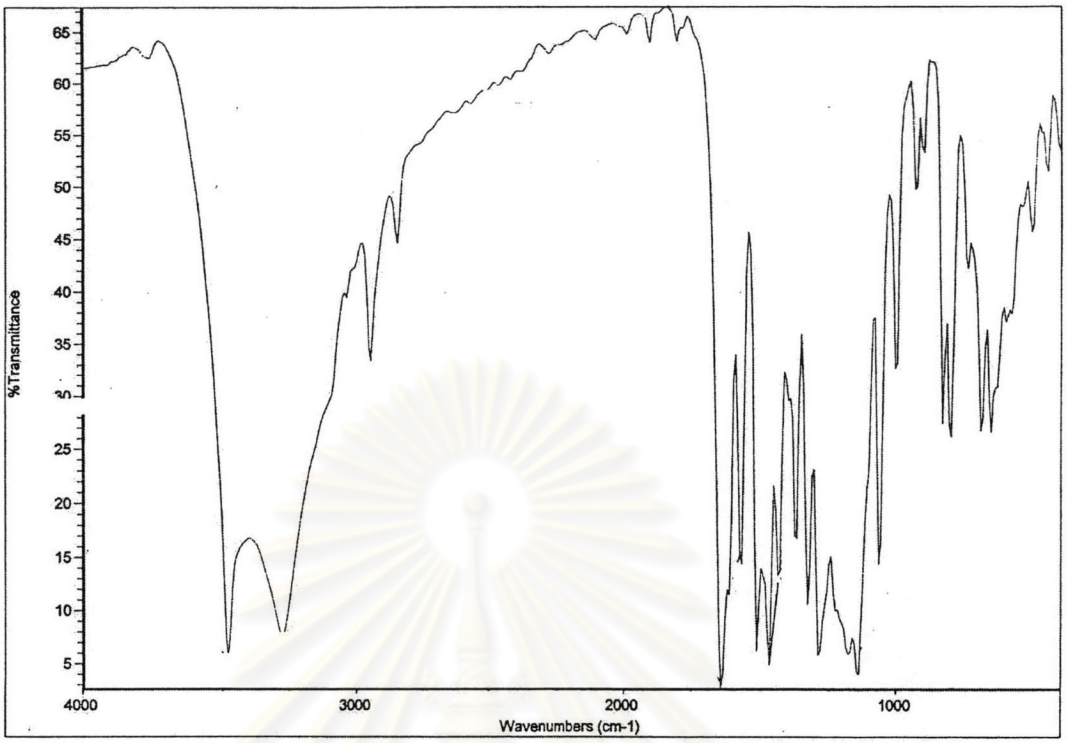


Figure 3.20 IR spectrum of compound **4**

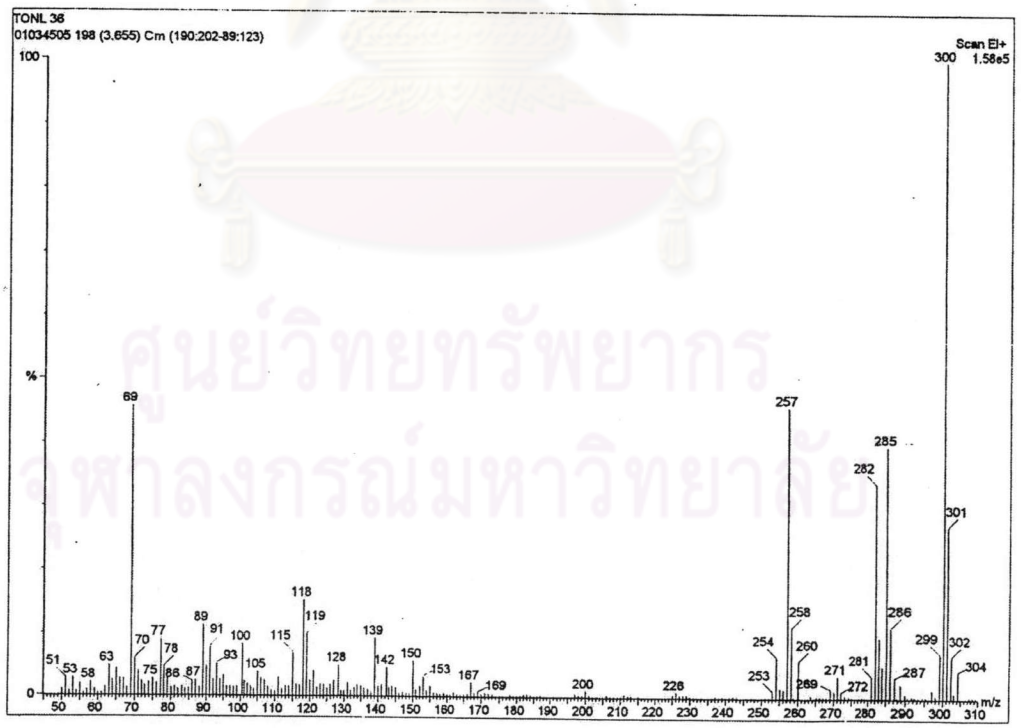


Figure 3.21 Mass spectrum of compound **4**

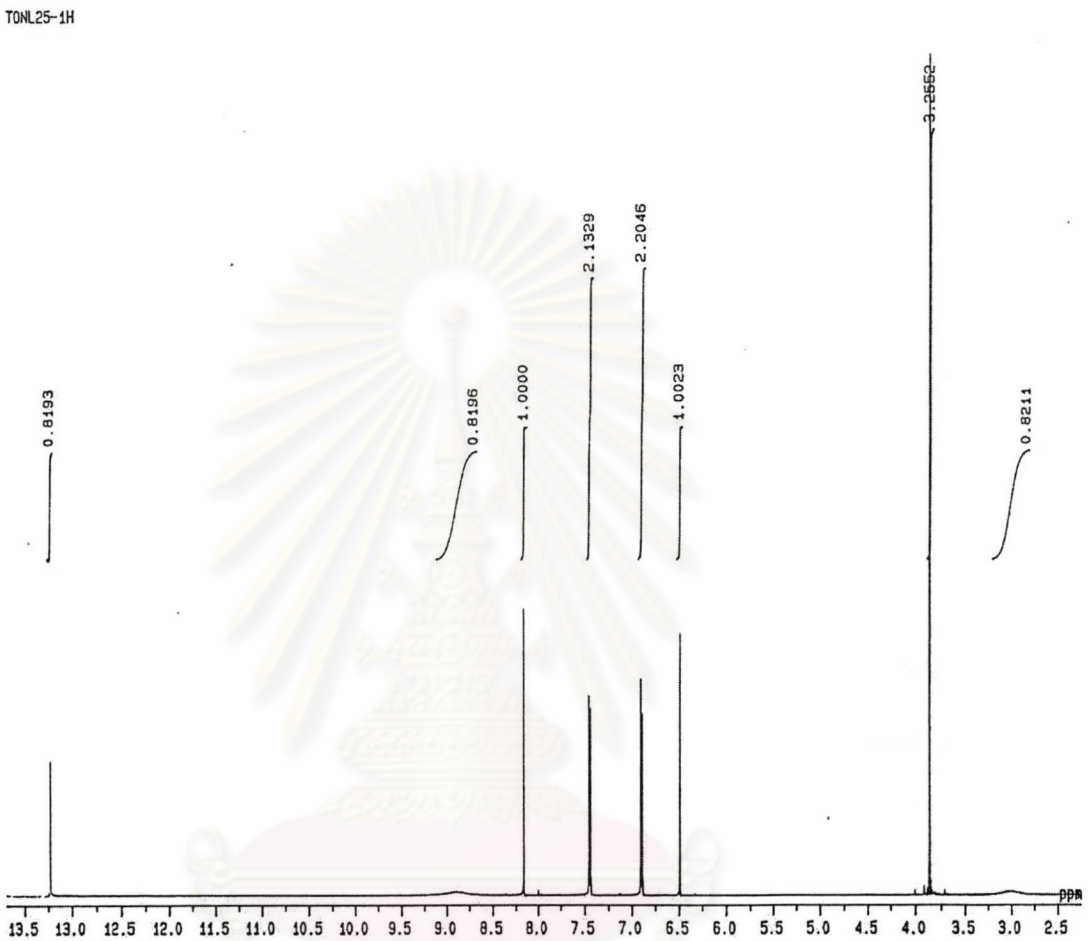


Figure 3.22 ^{1}H-NMR spectrum of compound **4**

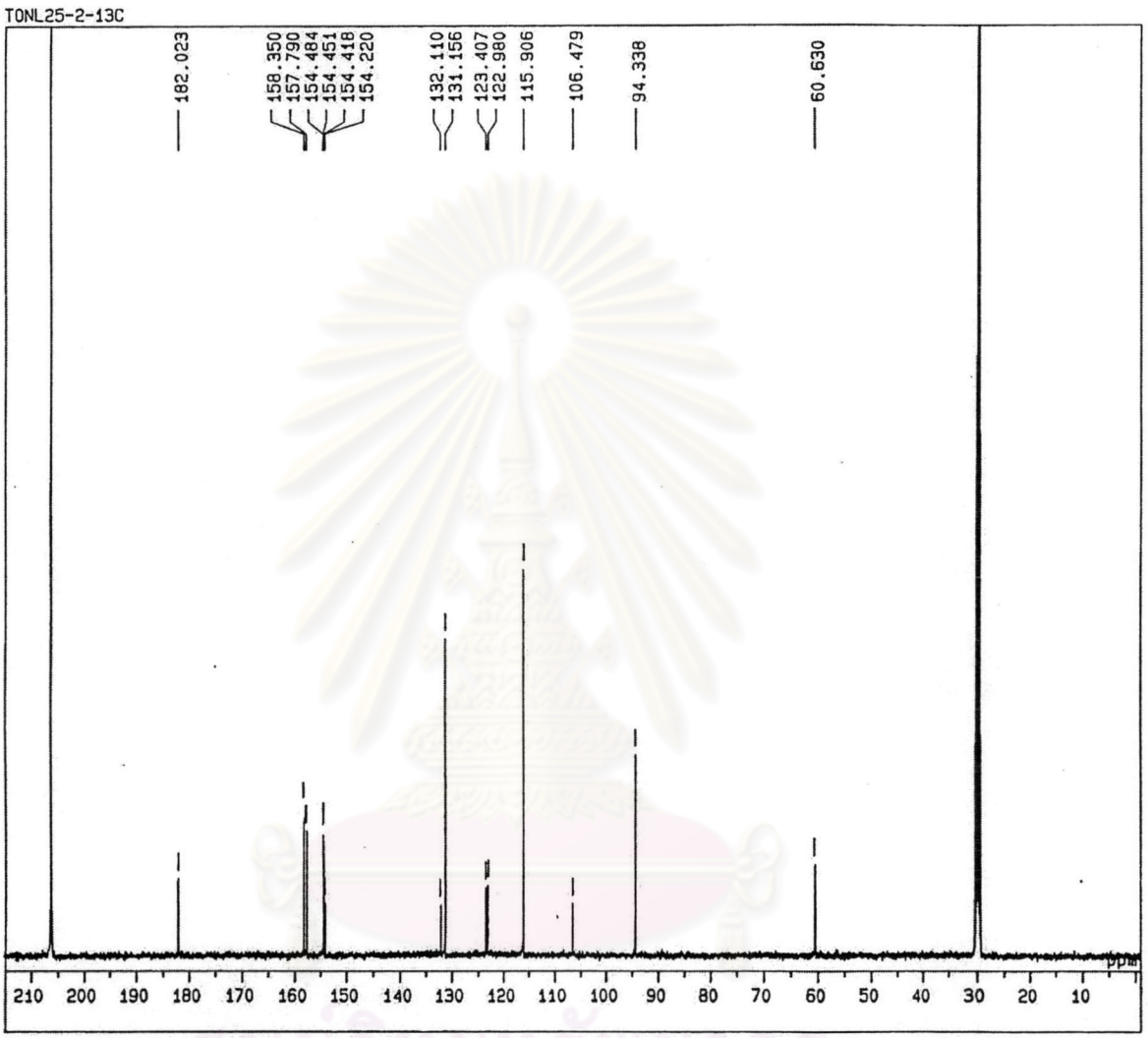


Figure 3.23 ^{13}C-NMR spectrum of compound **4**

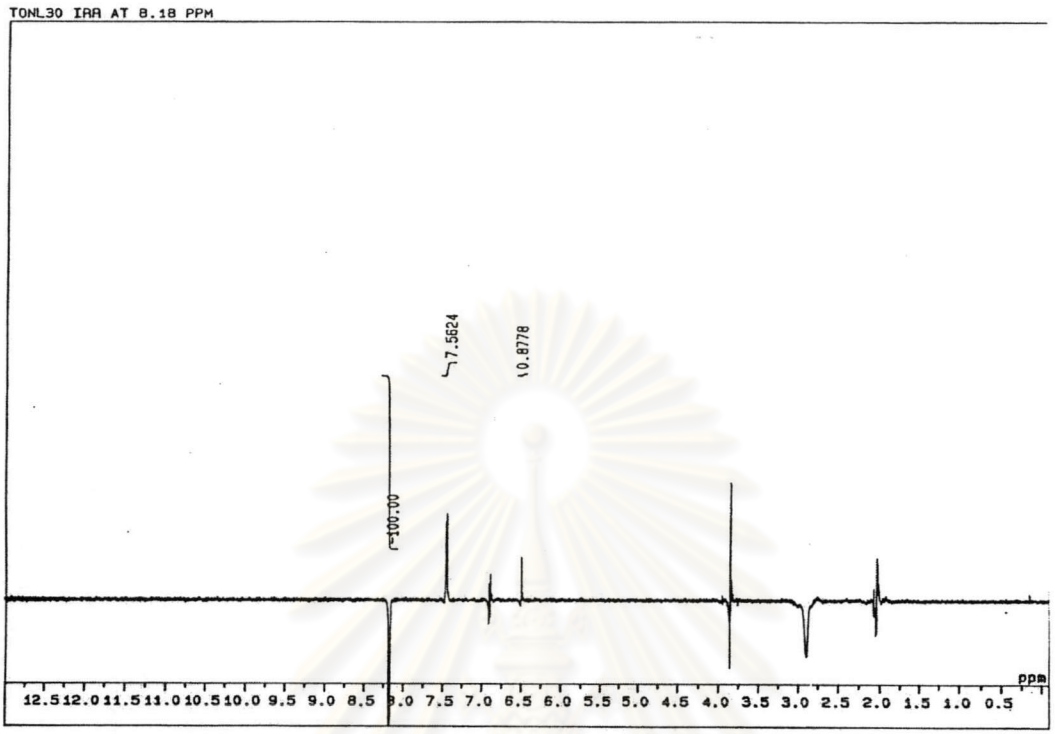


Figure 3.24 NOE DIFF of compound **4** (irradiated at 8.18 ppm)

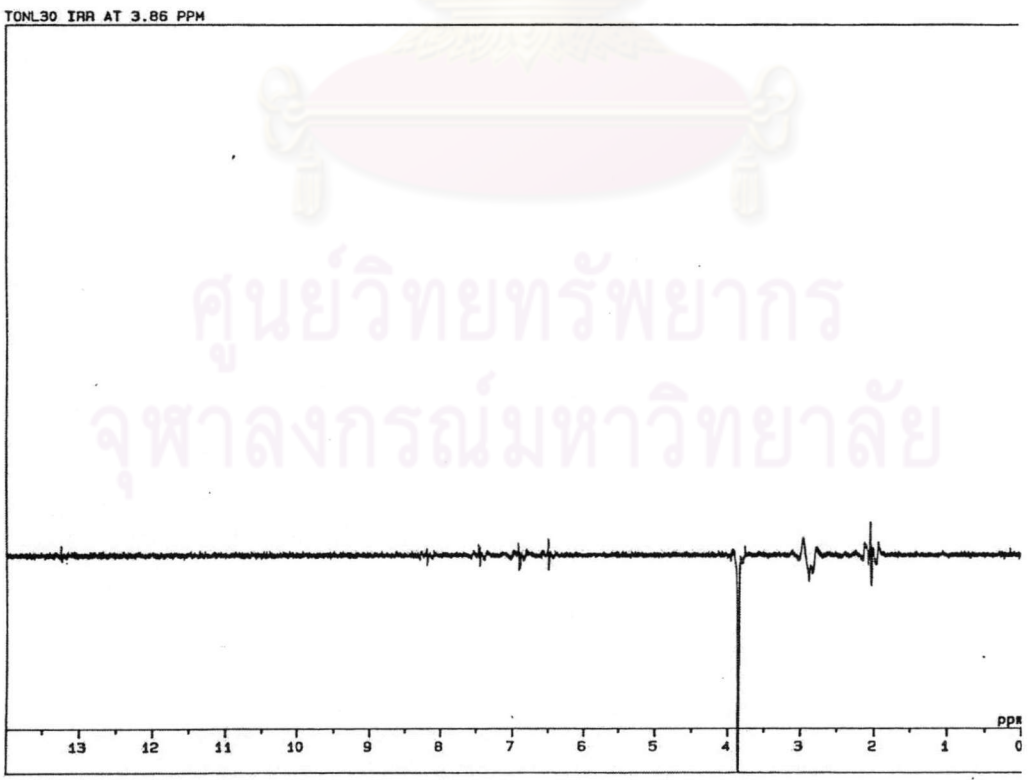


Figure 3.25 NOE DIFF of compound **4** (irradiated at 3.86 ppm)

3.2.5 Compound 5

Compound **5** was isolated from crude ethyl acetate extract and crystallized as colorless needles, mp. 246-248 °C. Its R_f value was 0.25 (SiO_2, EtOAc-CH_2Cl_2, 1:9) and was dissolved in hot methanol, unsoluble in ethyl acetate, dichloromethane and hexanes.

The IR absorption bands at 3200-3600 cm^{-1} indicated the presence of hydroxyl group (ν_{max} 1640 cm^{-1}) and C=O group (ν_{max} 1640 cm^{-1}) and aromatic ring (ν_{max} 1500 and 1460cm^{-1}) (**Figure 3.26**).

The EI mass spectrum exhibited a [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 3.27**), 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 spectral data.

The ^1H NMR spectrum (acetone-d_6, 500 MHz) of this compound showed seven aromatic protons, along with one methoxy group signal (δ 3.82). Two ortho protons coupled to each other were assigned to H-5 (δ 8.05, d, J = 8.2 Hz) and H-6 (δ 6.95, dd, J = 2.1, 8.2 Hz); meta coupled of H-8 (δ 6.85, d, J = 2.1 Hz) in ring A. The para-substituted of four aromatic protons at δ 7.46 (H-2', H-6', d, J = 8.2) and δ 6.97 (H-3', H-5', d, J = 8.2 Hz) in ring B. (**Figure 3.28**).

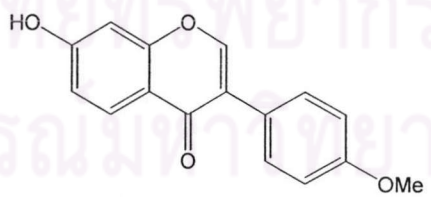
The ^{13}C NMR spectra (**Figure 3.29**) and Dept 90 and 135 (**Figure 3.30**) indicated sixteen carbons of fifteen skeleton and one methoxy group (flavonoid characteristic). This compound contained six quaternary carbons at δ 164.9 (C-7), 154.7 (C-9), 118.1 (C-10), 125.7 (C-3), 125.6 (C-1') and 158.4 (C-4'), eight methine carbons at δ 128.4 (C-5), 116.6 (C-6), 103.3 (C-8), 161.1 (C-2), 131.4 (C-2'), 114.9 (C-3'), 114.9 (C-5') and 131.4 (C-6'), one methoxy carbon and one carbonyl carbon at δ 178.0 (C-4) as showed in **Table 3.10**.

The NOE DIFF experiment was used to confirm the substitution pattern on ring B. The NOE DIFF spectra exhibited the methoxy group substituted at C-4' (**Figure 3.31**).

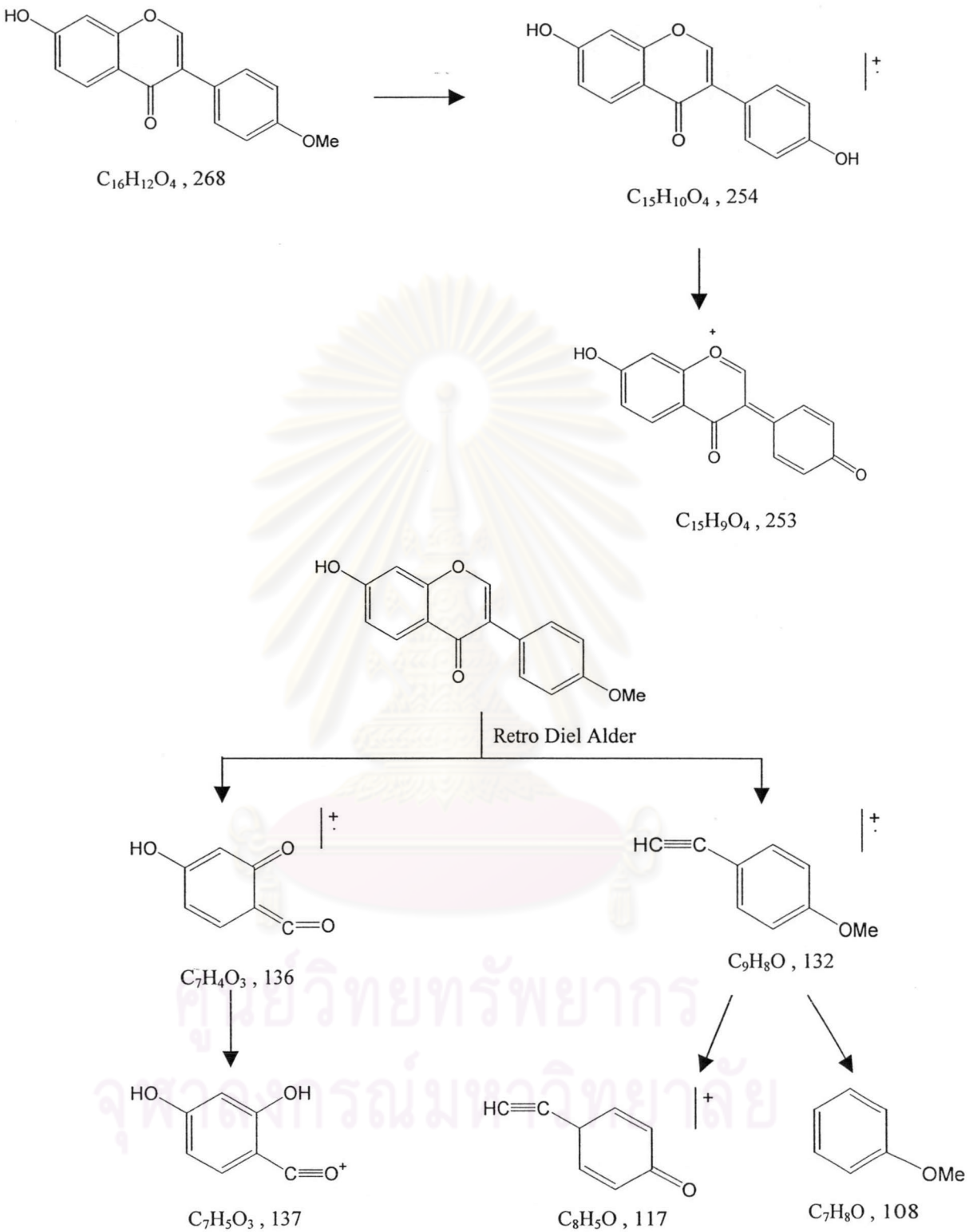
Table 3.10 ^{1}H and ^{13}C spectral data of compound **5**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	161.1	8.15	1H, s
3	125.7		
4	178.0		
5	128.4	8.05	1H, d, J = 8.1 Hz
6	116.6	6.95	1H, dd, J = 2.1, 8.2 Hz
7	164.9		
8	103.3	6.85	1H, d, J = 2.1 Hz
9	154.7		
10	118.1		
1'	125.6		
2'	131.4	7.46	2H, d, J = 8.2 Hz
3'	114.9	6.97	2H, d, J = 8.2 Hz
4'	158.4		
5'	115.9	6.97	2H, d, J = 8.2 Hz
6'	131.0	7.46	2H, d, J = 8.2 Hz

Based on all spectral data, the structure of compound **5** was identified as 7-hydroxy-4'-methoxyisoflavone or formononetin.[21]



The structure of compound **5** (formononetin)



Scheme 3.5 Possible mass fragmentation patterns of compound **5**

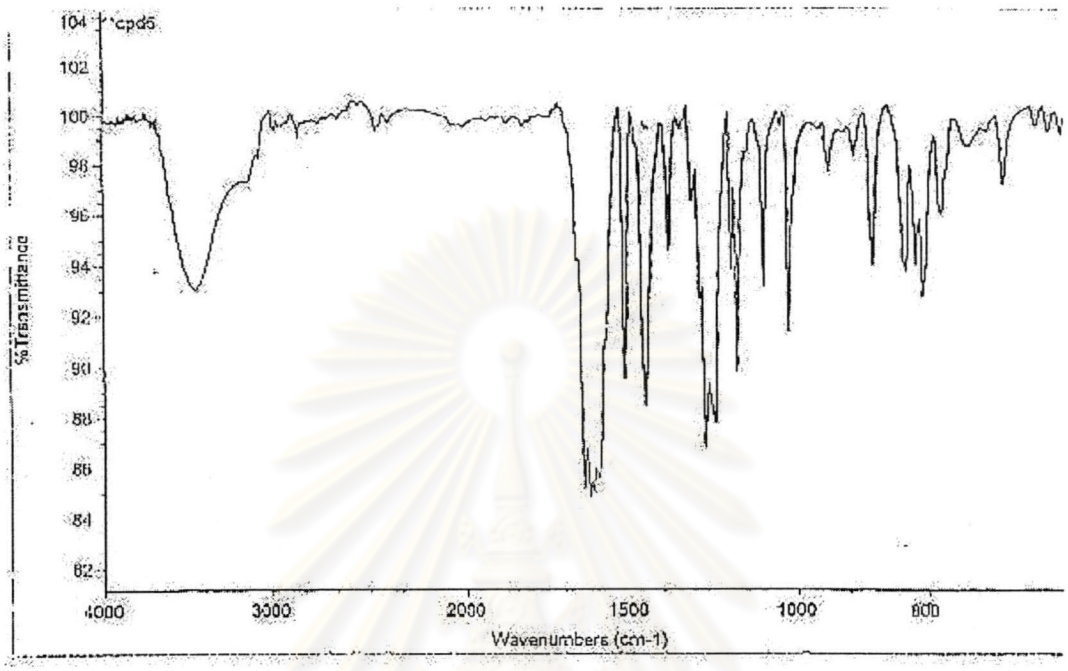


Figure 3.26 IR spectrum of compound **5**

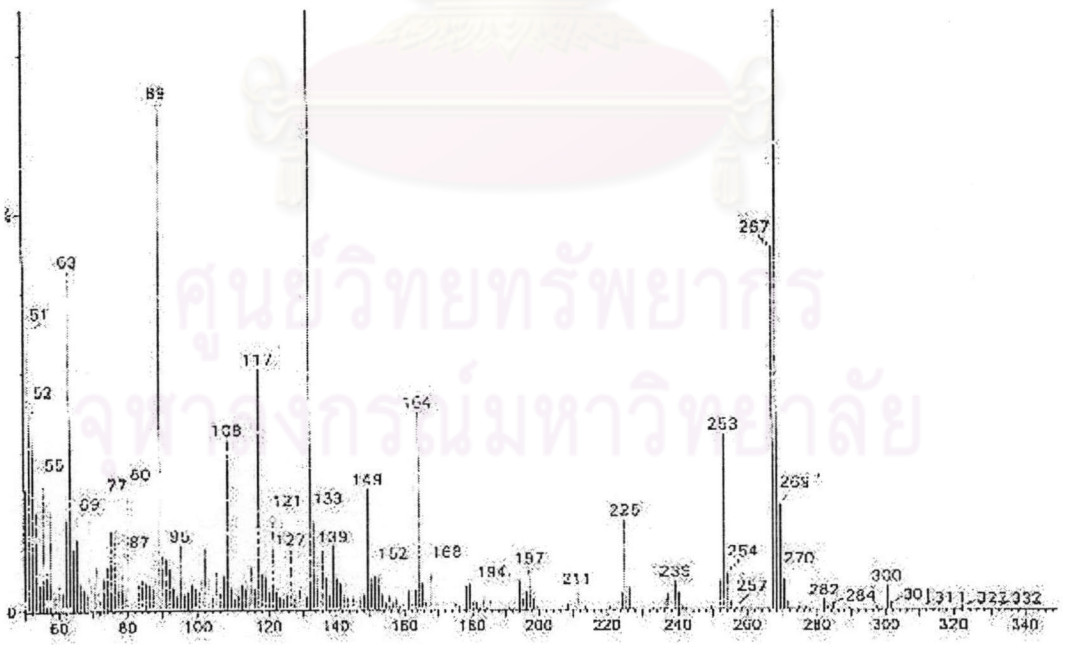


Figure 3.27 Mass spectrum of compound **5**

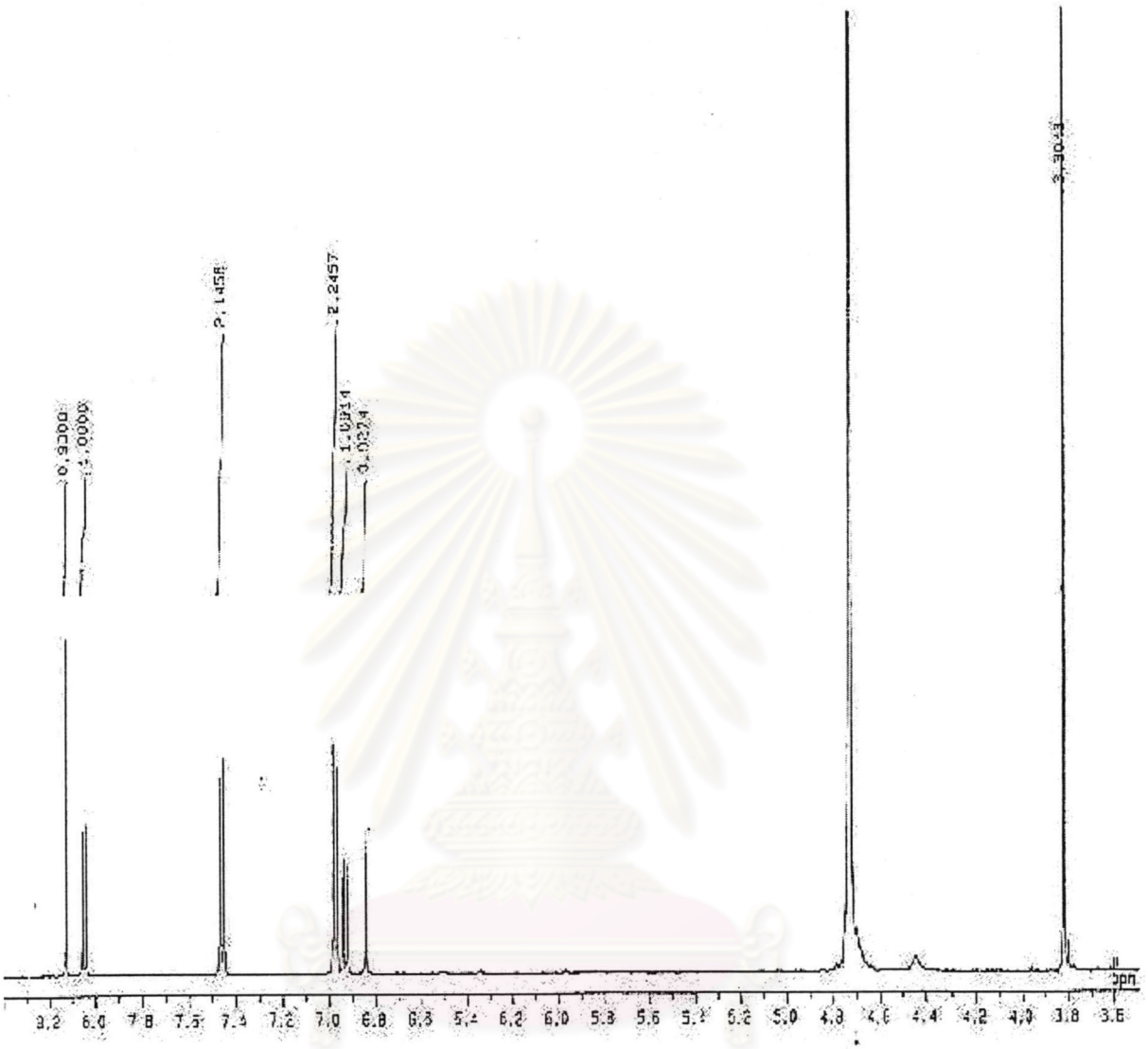


Figure 3.28 ^{1}H-NMR spectrum of compound **5**

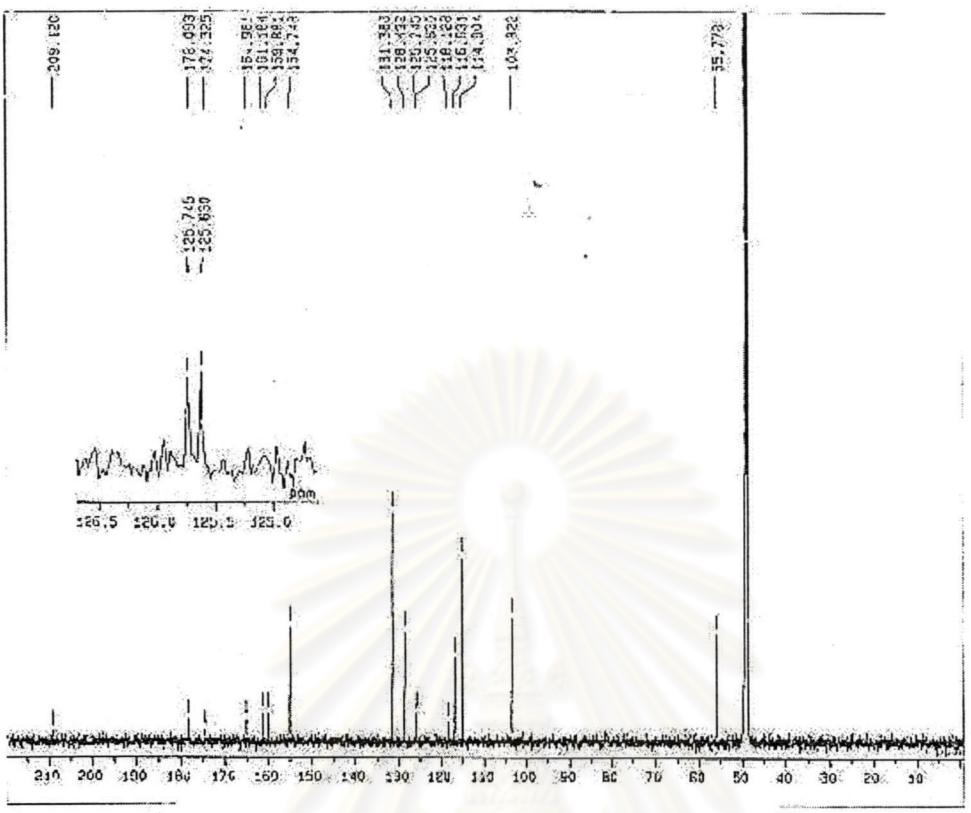


Figure 3.29 ^{13}C-NMR spectrum of compound **10**

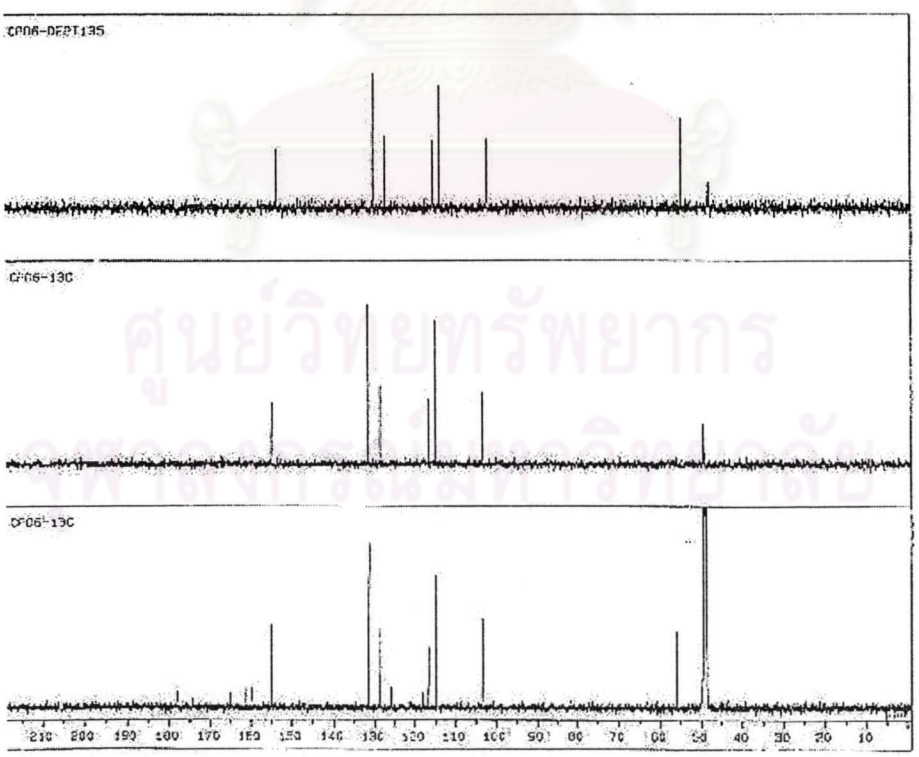


Figure 3.30 DEPT 90 and 135 spectrums of compound **5**

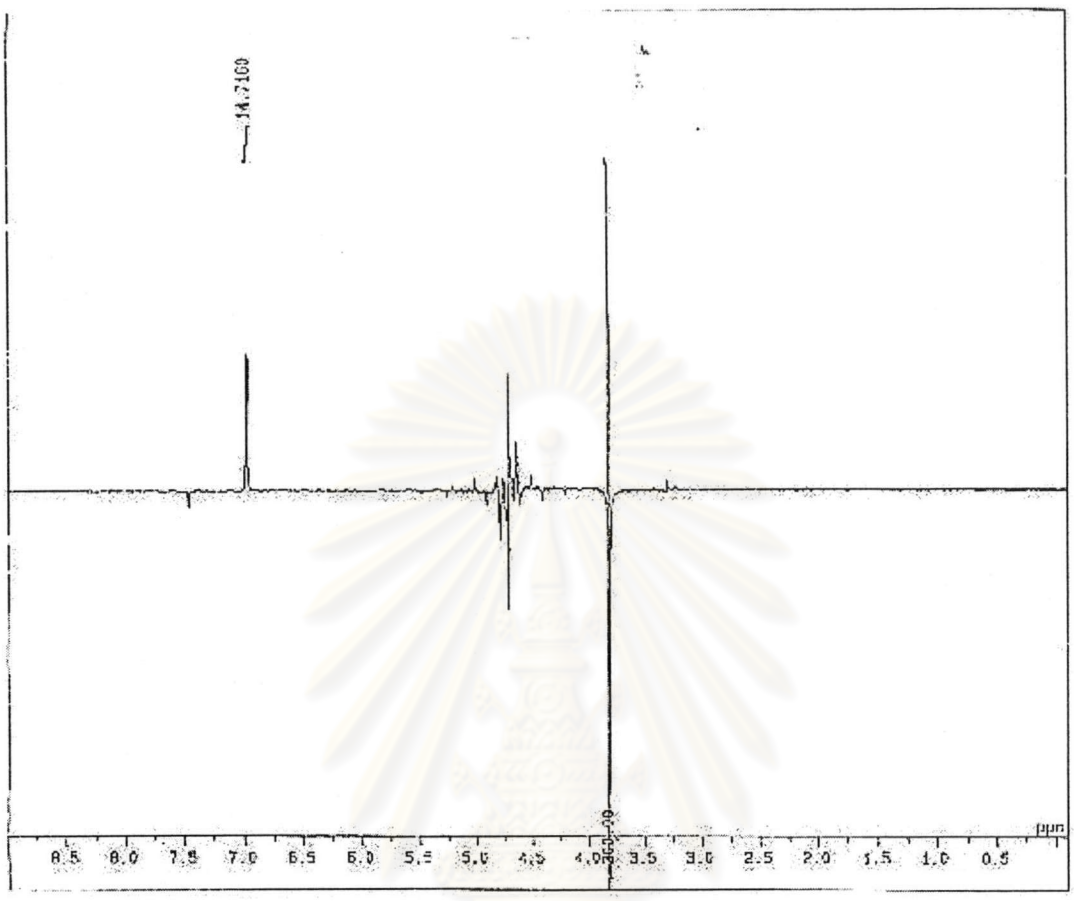


Figure 3.31 NOE DIFF of compound **5** (irradiated at 3.81 ppm)

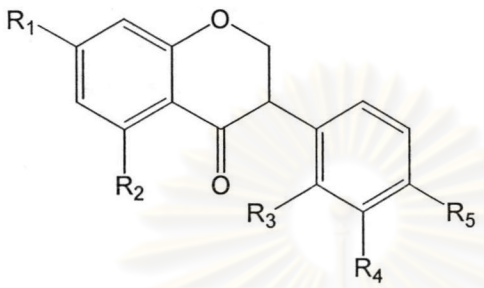
3.2.6 Compound 6

Compound **6**, a colorless amorphous powder with m.p. 199-202 °C and R_f value 0.7(SiO_2, EtOAc-CH_2Cl_2,6:4), was purified by column chromatography of ethyl acetate crude extract. The molecular formula of compound **6** was assigned as $C_{17}H_{16}O_7$ on the basis of EIMS, ^1H, and ^{13}C-NMR data and gave [M^+] at m/z 332 in EI-MS (**Figure 3.33**) together with fragment ions at m/z 180, 153, 165 and 133. Its IR spectrum (**Figure 3.32**) indicated the presence of hydroxyl groups (ν_{max} 3359 cm^{-1}), carbonyl group (ν_{max} 1639 cm^{-1}) and benzene rings (ν_{max} 1603, 1501 and 1490 cm^{-1}).

The ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.34**) showed a set of mutually coupled three protons [δ 4.43 (dd, J = 11, 6 Hz, H-2), 4.53 (dd, J = 11, 11 Hz, H-2) and 4.25 (dd, J = 11, 6 Hz, H-3)], clarifying that compound **6** had an isoflavanone skeleton. The presence of three hydroxyls [δ 8.00, 9.91 and 12.35 (chelated)] and two methoxy groups (δ 3.81, 3.82) were supported by the ^1H-NMR spectrum and significant fragment ions [m/z 153 and 180] caused by the retro Diels Alder fragmentation. Two hydroxyl groups were located on the A ring and one hydroxyl and two methoxyl groups in ring B. The chelated hydroxyl group was assigned to the C-5 and indicated a set of ortho-coupled protons (δ 6.65 , H-5' and 6.69, H-6') and meta-coupled protons (δ 5.95, H-8 and 5.97, H-6).

The ^{13}C-NMR spectrum (**Figure 3.35**) showed seventeen carbon signals of fifteen skeletons and two substituent groups (characteristic of flavonoid); two methoxy groups at δ 56.4 (-OMe) and 60.1 (OMe); five methine carbons at δ 48.0 (C-3), 96.9 (C-6), 95.6 (C-8), 107.3 (C-5') and 120.4 (C-6'); one methylene carbon at δ 71.4 and nine quaternary carbons at δ 198.3 (C-4, C=O), 165.6 (C-5), 167.0 (C-7), 164.5 (C-9), 103.3 (C-10), 122.0 (C-1'), 146.8 (C-2'), 140.3 (C-3') and 149.4 (C-4').

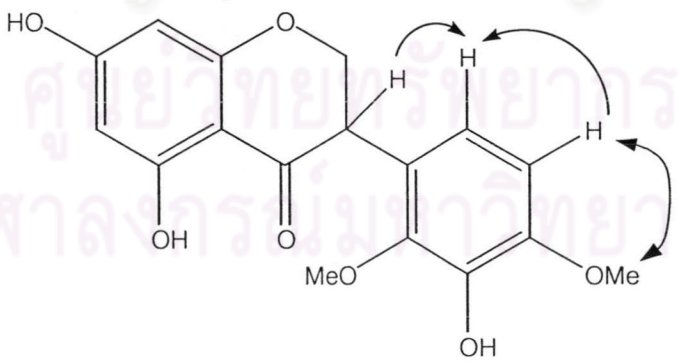
All of the above data suggested the possibility of a flavonoid skeleton of isoflavanone type. Therefore, the data were comparison with ^{1}H, ^{13}C-NMR of known isoflavanone suggested that the molecule of this compound must have the basic skeleton as shown follow :



Substituents :

- OH (3)
- OMe (2)

Irradiation of the proton signal at δ 6.69 resulted in NOE enhancement of the proton signal at δ 6.65 (H-6') and 3.83 (OMe), whereas irradiation of the proton signal at δ 4.25 only caused enhancement of the proton signal at δ 6.65 (H-6'). On the other hand, irradiation of methoxy protons at δ 3.83 had enhancement effect on the proton signal at δ 6.69 (H-5') (**Figures 3.36-3.39**). The complete assignment of compound **6** was indicated in **Table 3.11**.



NOE DIFF correlation of compound **6**

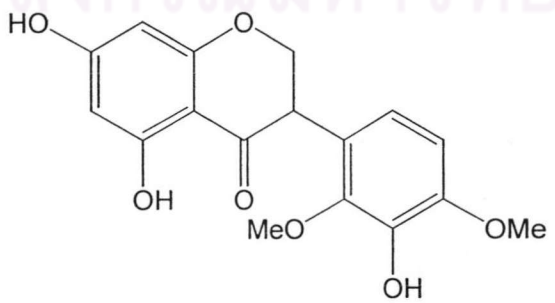
Table 3.11 ^{1}H and ^{13}C spectral data of compound **6**

Position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	71.4	4.59-4.29	2H, m
3	48.0	4.26	1H, m
4	198.3		
5	165.6		
6	96.9	5.97	1H, d, J = 2.1 Hz
7	167.0		
8	95.6	5.95	1H, d, J = 2.1 Hz
9	164.5		
10	103.3		
1'	122.0		
2'	146.8		
3'	140.3		
4'	149.4		
5'	107.3	6.69	1H, d, J = 8.5 Hz
6'	120.4	6.65	1H, d, J = 8.5 Hz
-OMe	56.4	3.81(C-4')	3H, s
-OMe	60.1	3.83(C-2')	3H, s

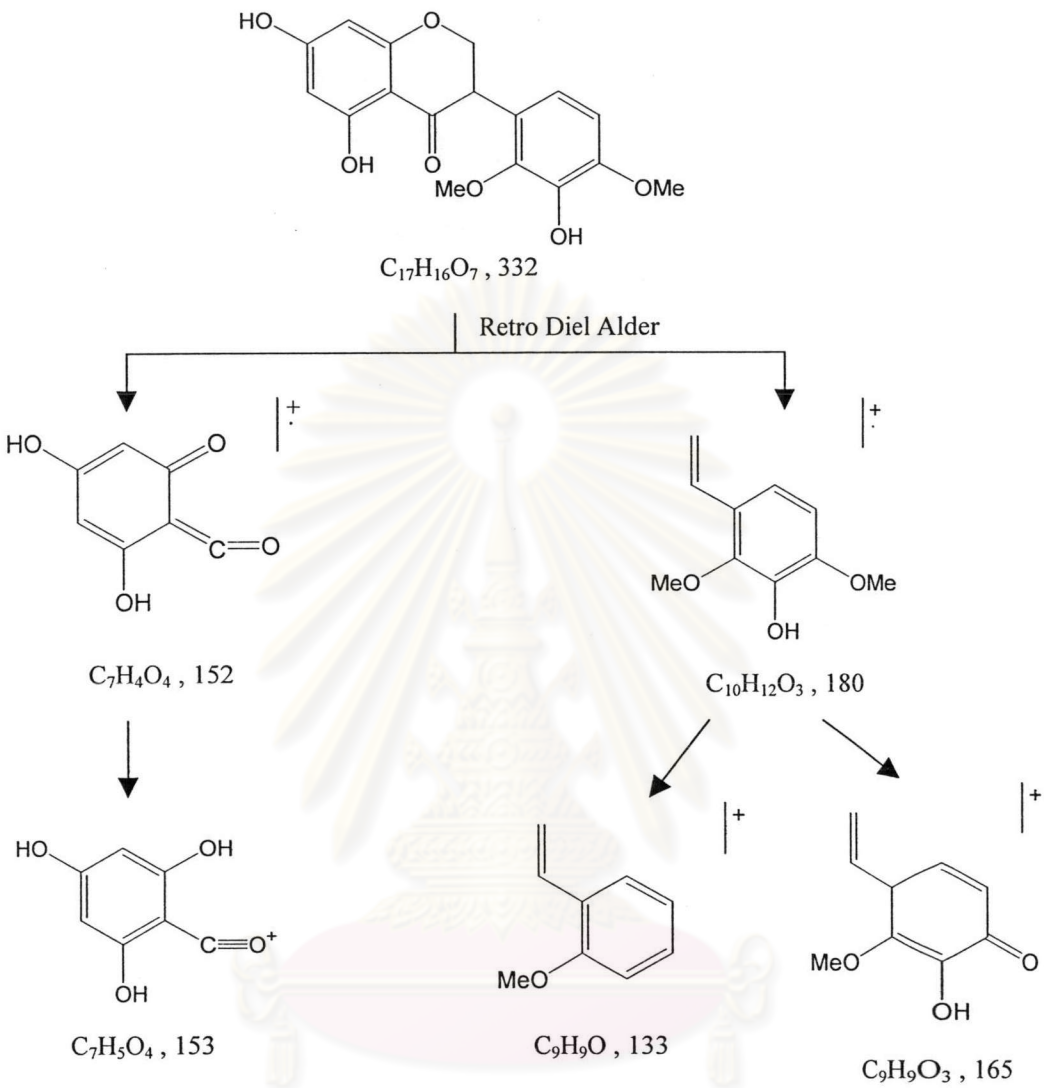
Table 3.12 ^{13}C NMR data of compound **6** compared with Secundiflorol H.[24]

Position	Compound 6	Secundiflorol H
2	71.4	71.4
3	48.0	48.1
4	198.3	198.2
5	165.6	165.7
6	96.9	96.9
7	167.0	169.1
8	95.6	95.6
9	164.5	164.5
10	103.3	103.4
1'	122.0	122.1
2'	146.8	146.8
3'	140.2	140.3
4'	149.3	149.4
5'	107.3	107.3
6'	120.4	120.4
-OMe	56.4	56.5
-OMe	60.1	60.1

On the basis of spectroscopic observation and a comparison of the ^{13}C-NMR spectrum of compound **6** with published data of secundiflorol H, the structure of compound **6** was elucidated as 5,7,3'-trihydroxy-2',4'-dimethoxyisoflavanone or secundiflorol H.



The structure of compound **6** (secundiflorol H)



Scheme 3.6 Possible mass fragmentation patterns of compound **6**

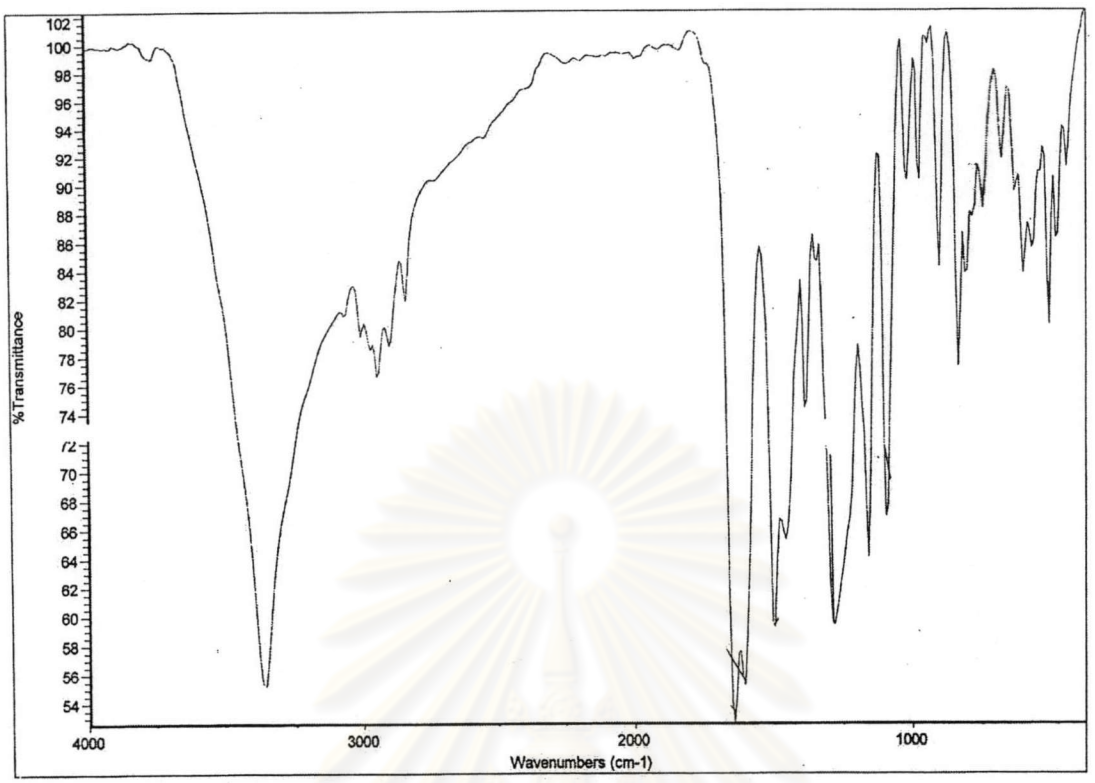


Figure 3.32 IR spectrum of compound **6**

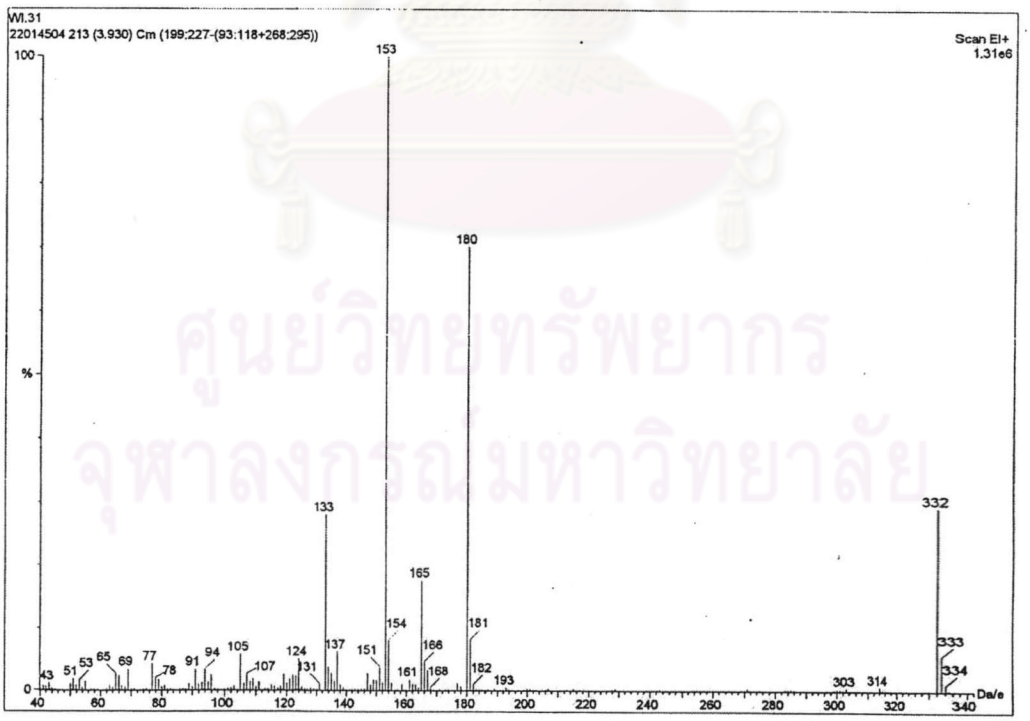


Figure 3.33 Mass spectrum of compound **6**

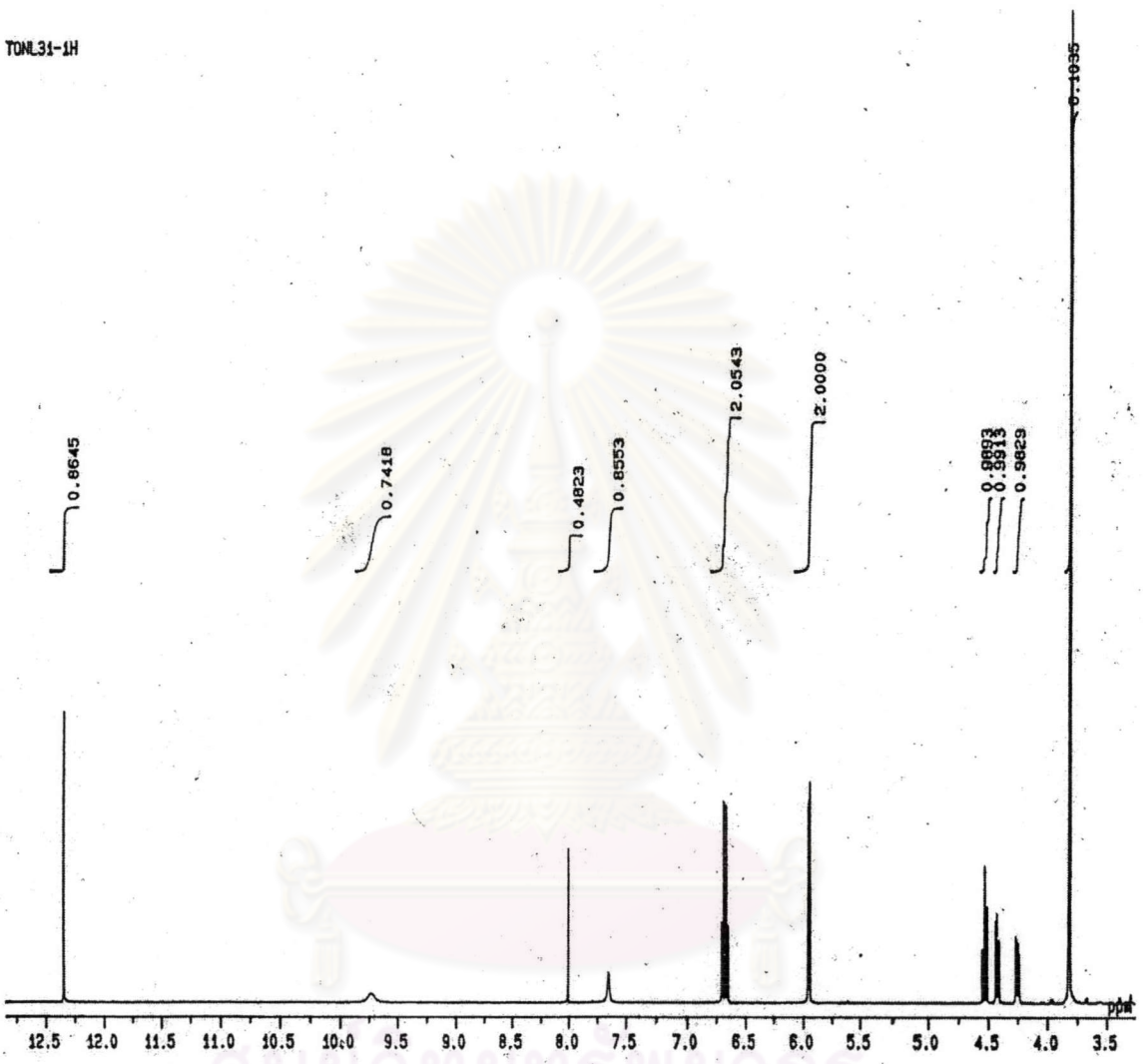


Figure 3.34 ^{1}H-NMR spectrum of compound **6**

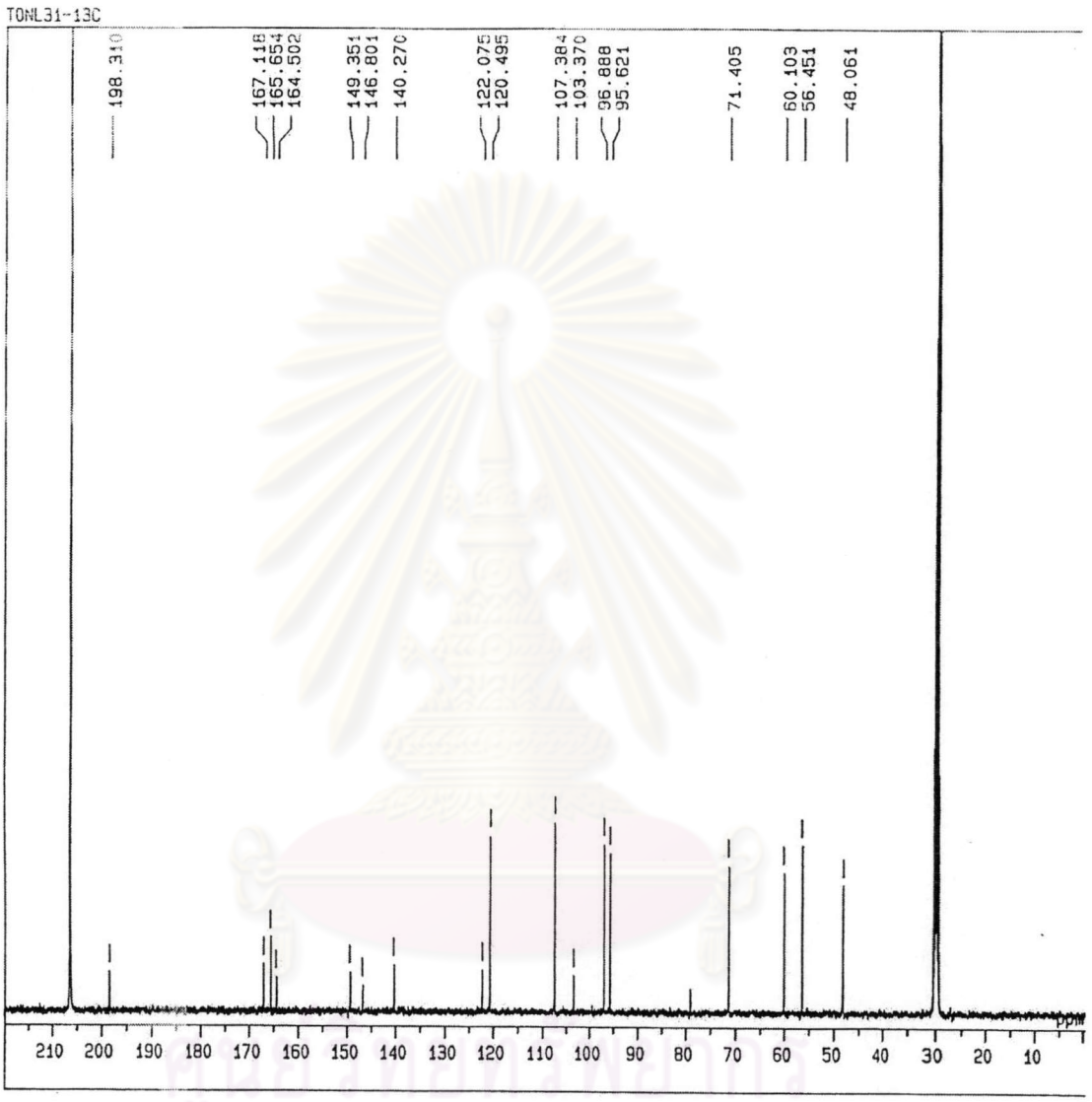


Figure 3.35 ^{13}C-NMR spectrum of compound **6**

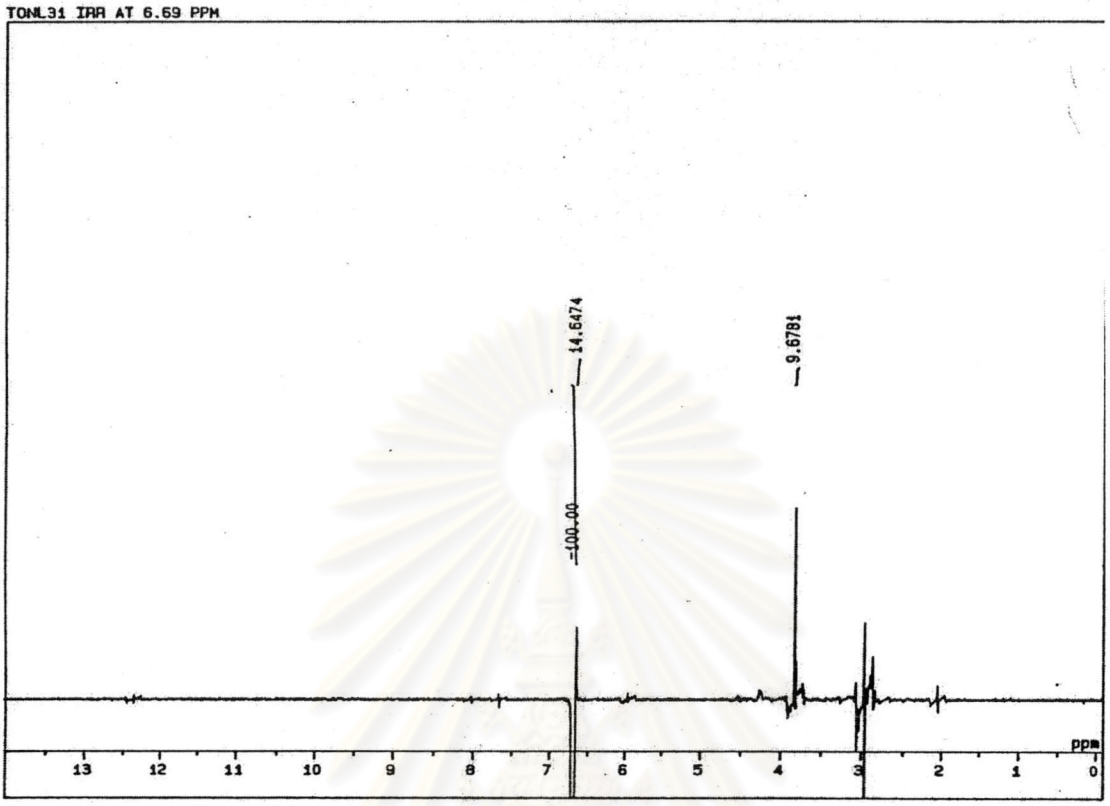


Figure 3.36 NOE DIFF of compound **6** (irradiated at 6.69 ppm)

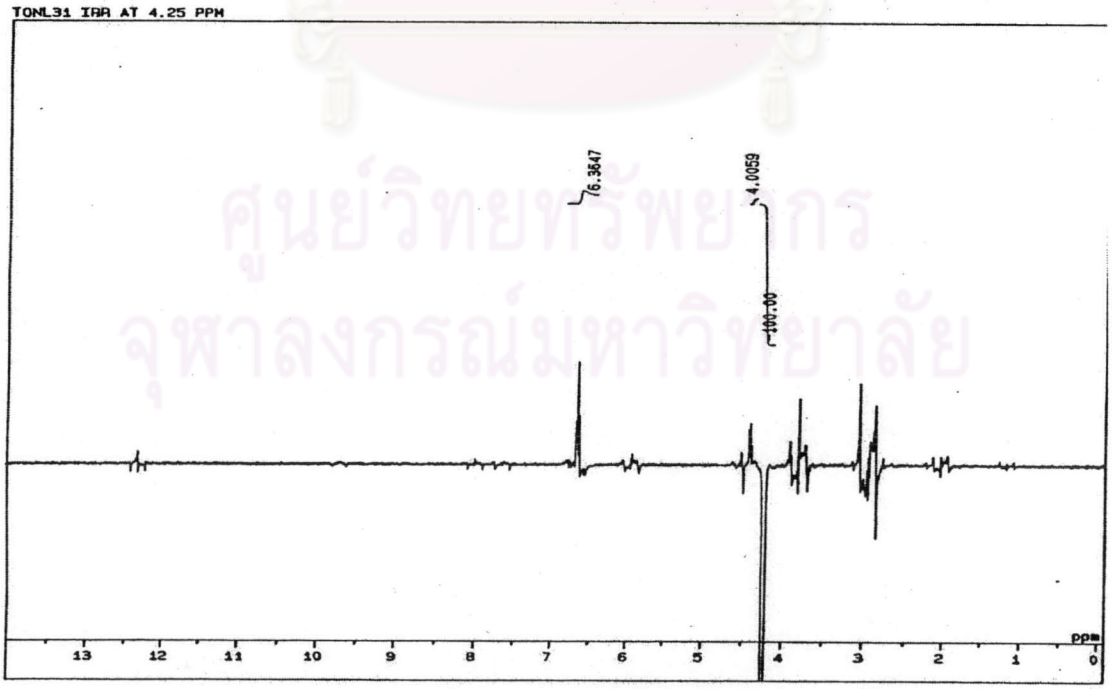


Figure 3.37 NOE DIFF of compound **6** (irradiated at 4.25 ppm)

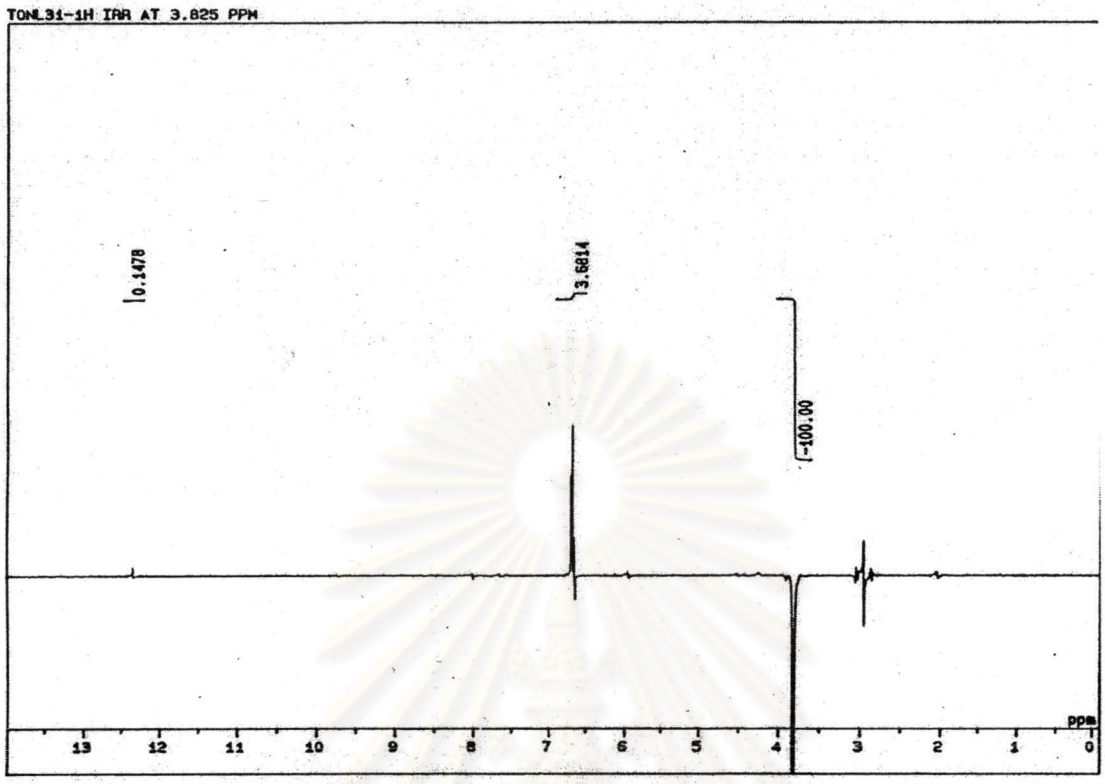


Figure 3.38 NOE DIFF of compound **6** (irradiated at 3.825 ppm)

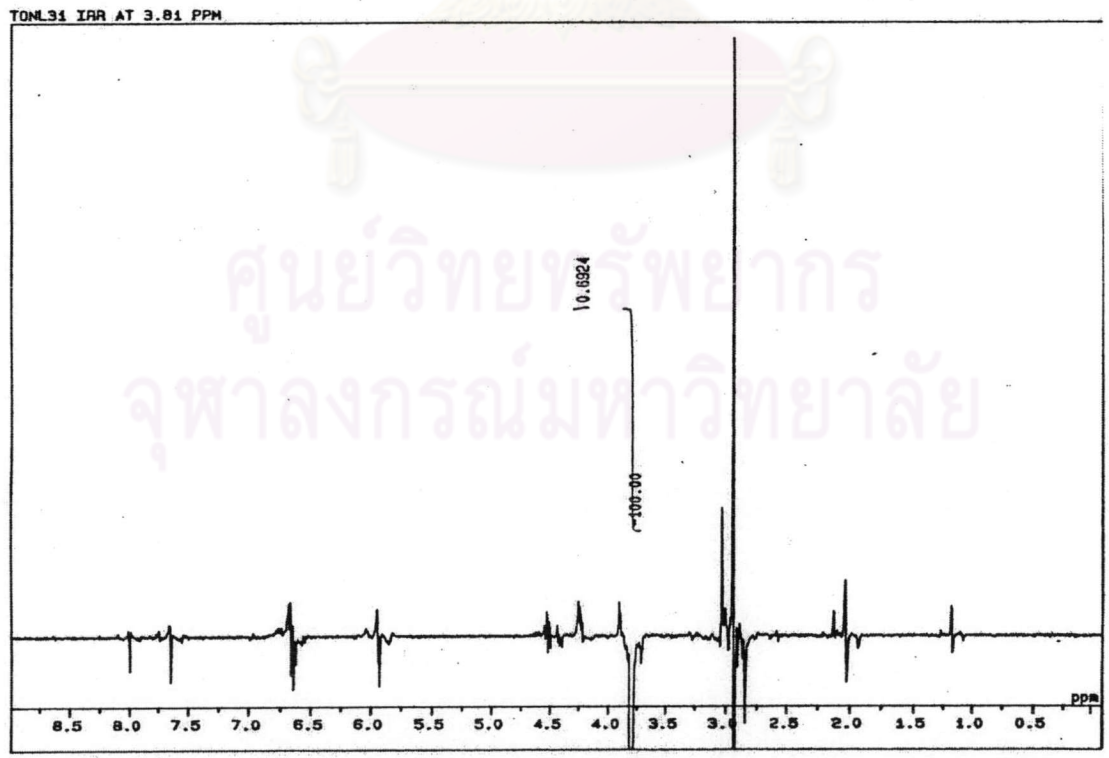


Figure 3.39 NOE DIFF of compound **6** (irradiated at 3.81 ppm)

3.2.7 Compound 7

Compound **7** was isolated by column chromatography of the crude ethyl acetate extract and further purified by recrystallization in acetone. This compound, m.p. 174-176 °C, was obtained as brown cubes (0.2 g) and soluble in acetone, methanol and slightly in ethyl acetate. The R_f value was 0.68 (SiO_2, EtOAc-CH_2Cl_2 , 6:4). The mass spectrum (**Figure 3.41**) gave a molecular ion $[M^+]$ peak at m/z 286 along with fragment ion peaks at m/z 166, 164, 135 and 123, consistent with a molecular formula of $C_{16}H_{14}O_5$.

The IR spectrum (**Figure 3.40**) displayed absorption bands of hydroxy at ν_{max} 3482 cm^{-1}, a carbonyl group which conjugated with double bond at ν_{max} 1644 cm^{-1} and aromatic ring at ν_{max} 1603, 1506 and 1449 cm^{-1}.

The ^{1}H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.42**) displayed a set of mutually coupled five protons [δ 2.81 (2H, m), 3.35 (1H, m) and 3.99-4.24 (2H, m)] suggested the presence of a partial structure of $-CH_2CH(Ar)CH_2O-$ assigned to H-4, H-3 and H-2 in an isoflavan skeleton. This spectrum also revealed five aromatic hydrogen signals at δ 6.89 (1H, d, J = 8.2 Hz), 6.38 (1H, dd, J = 2.4, 8.2 Hz), 6.27 (1H, d, J = 2.4 Hz), 6.50 (1H, s) and 6.06 (1H, d, J = 1.2 Hz) on ring A and B along with one aromatic methoxy signal at δ 3.84 (3H, s).

The ^{13}C-NMR spectrum (**Figure 3.43**) revealed sixteen carbon signals of fifteen carbon skeletons and one substituted group (flavonoid characteristic). The analysis of this spectrum by the aid of DEPT 90 and 135 techniques (**Figure 3.44**) unequivocally indicated that compound **7** contained seven quaternary carbons at δ 157.7 (C-7), 155.6 (C-9), 112.5 (C-10), 149.6 (C-1'), 187.3 (C-2', C=O), 159.5 (C-4') and 182.4 (C-5', C=O); six methine carbons at δ 31.9 (C-3), 131.4 (C-5), 109.3 (C-6), 103.6 (C-8), 108.5 (C-3') and 131.0 (C-6'); two methylene carbons at δ 69.0 (C-2) and 28.0 (C-4) and one methoxy group at δ 56.6 (C-4', OMe). The complete assignment of compound **7** was suggested on **Table 3.13.**

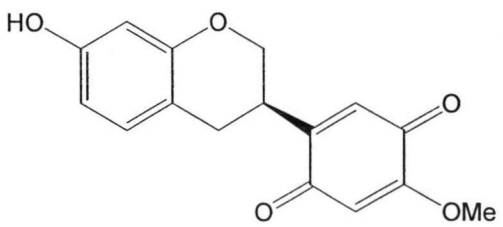
Table 3.13 ^{1}H and ^{13}C spectral data of compound **7**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	69.0	3.99-4.24	2H, m
3	31.9	3.35	1H, m
4	28.0	2.81	2H, m
5	131.4	6.89	1H, d, J = 8.2 Hz
6	109.3	6.38	1H, dd, J = 2.4, 8.2 Hz
7	157.7		
8	103.6	6.27	1H, d, J = 2.4 Hz
9	155.6		
10	112.5		
1'	149.6		
2'	187.3		
3'	108.5	6.50	1H, s
4'	159.5		
5'	182.4		
6'	131.0	6.06	1H, d, J = 1.2 Hz
-OMe	56.6	3.84	3H, s

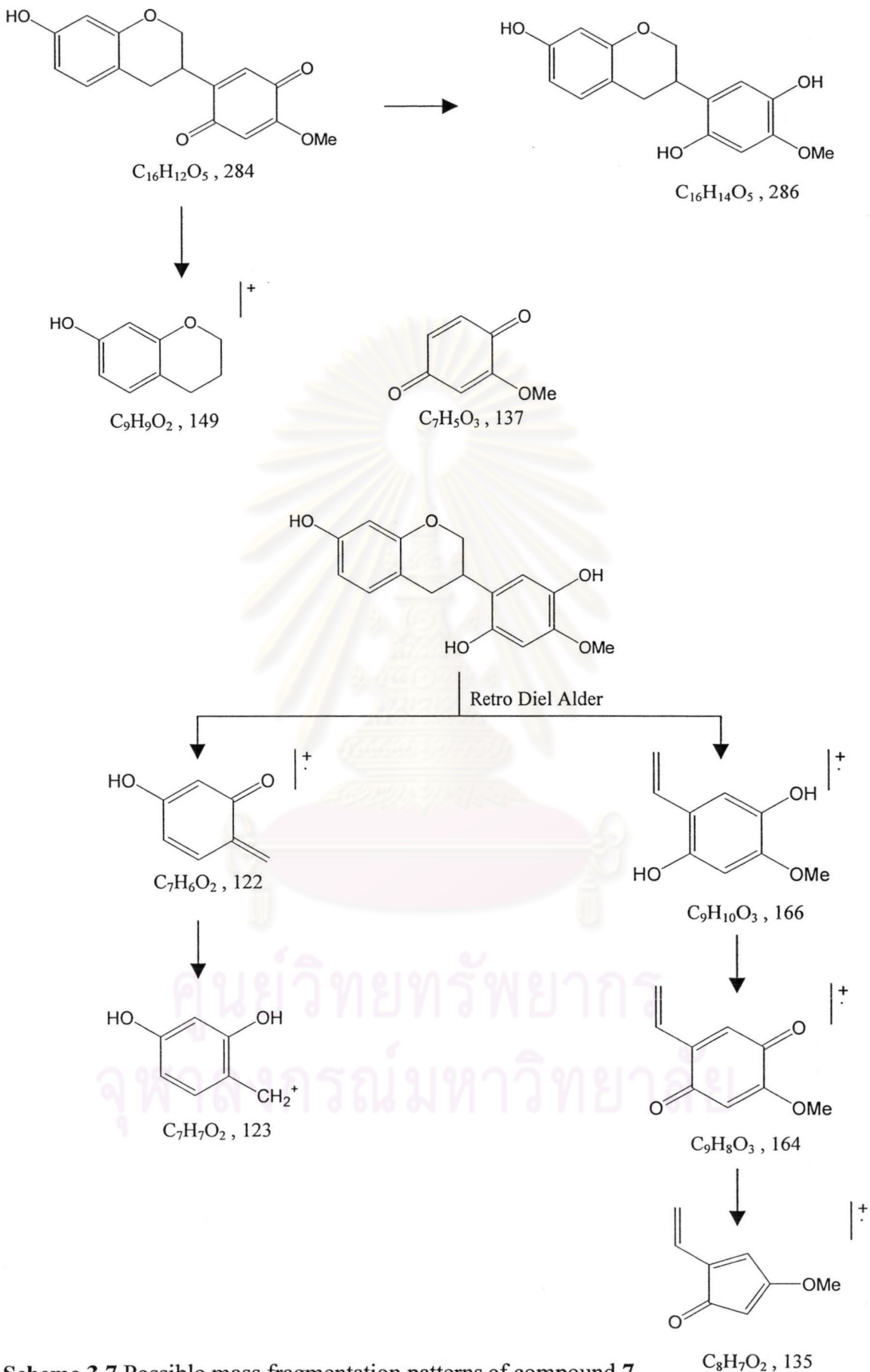
Table 3.14 ^{13}C NMR data of compound **7** compared with Claussequinone.[22]

Position	Compound **7**	claussequinone.
2	69.0	67.8
3	31.9	30.6
4	28	28.7
5	131.4	130.3
6	109.3	108.4
7	157.7	156.6
8	103.6	102.5
9	155.6	154.1
10	112.5	111.1
1'	149.6	148.3
2'	187.3	186.4
3'	108.5	107.5
4'	159.5	158.2
5'	182.4	181.6
6'	131.0	129.9
-OMe	56.6	56.3

Hence, on the basis of the interpretation of the above mentioned data and comparison with a previously reported data of claussequinone, the structure of compound **7** was elucidated as 2-methoxy-5-(7-hydroxychroman-3-yl)-1,4-benzoquinone or claussequinone.



Structure of compound **7** (claussequinone)



Scheme 3.7 Possible mass fragmentation patterns of compound **7**

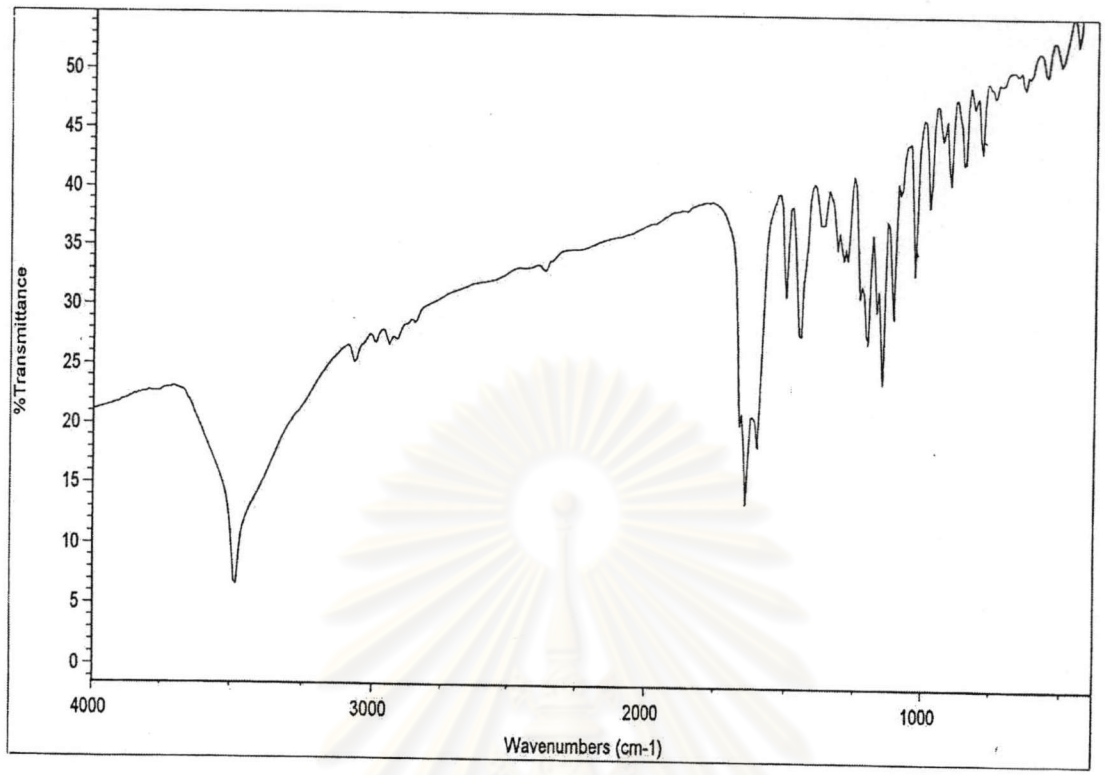


Figure 3.40 IR spectrum of compound **7**

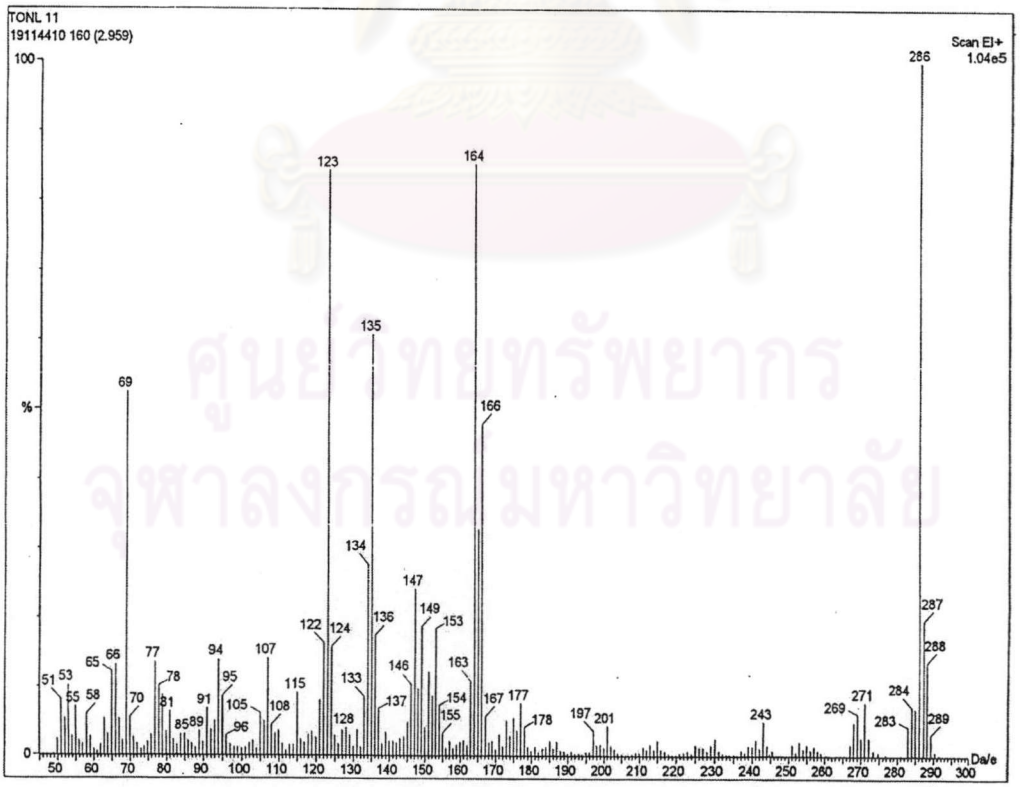


Figure 3.41 Mass spectrum of compound **7**

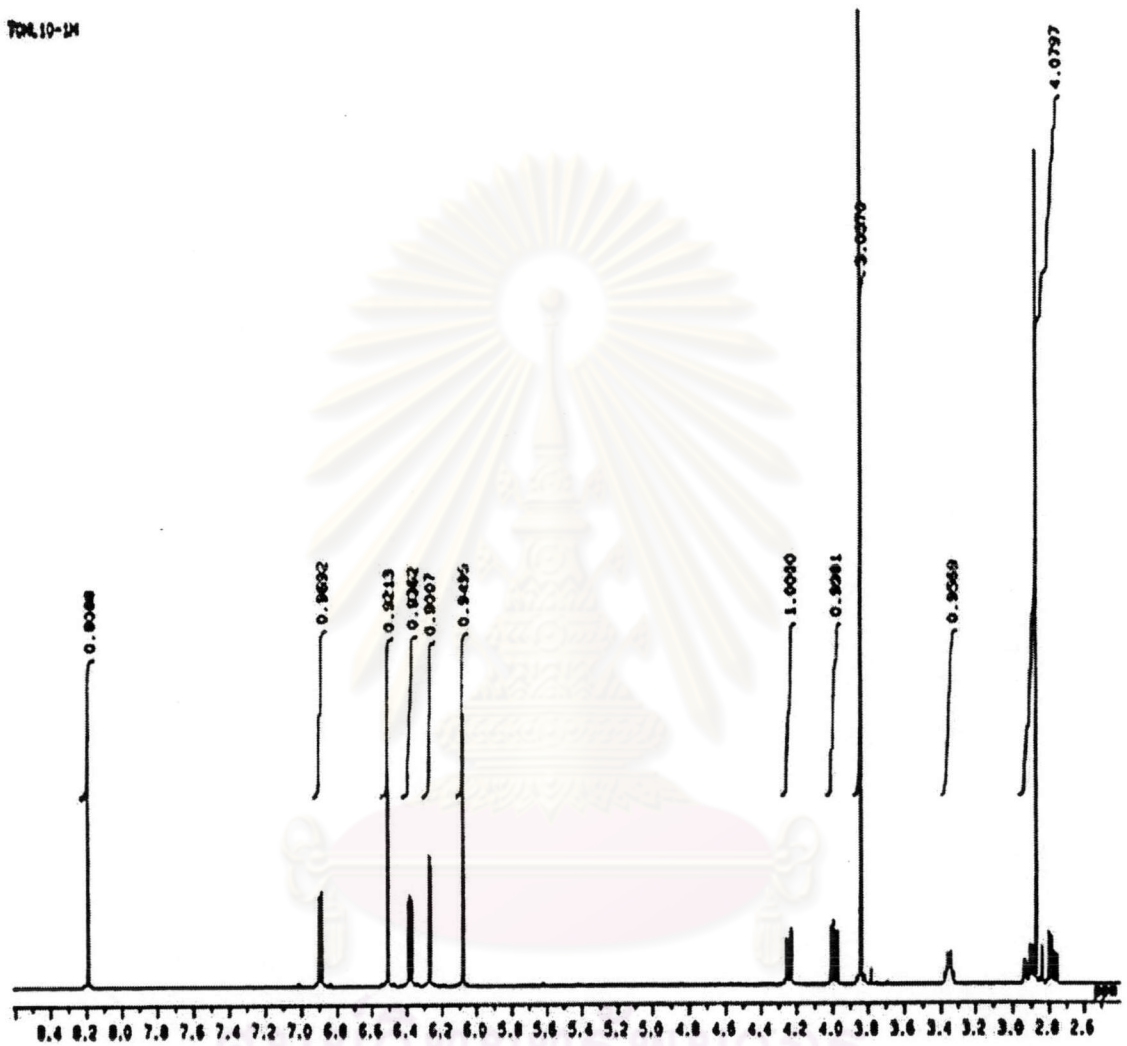


Figure 3.42 ^{1}H-NMR spectrum of compound **7**

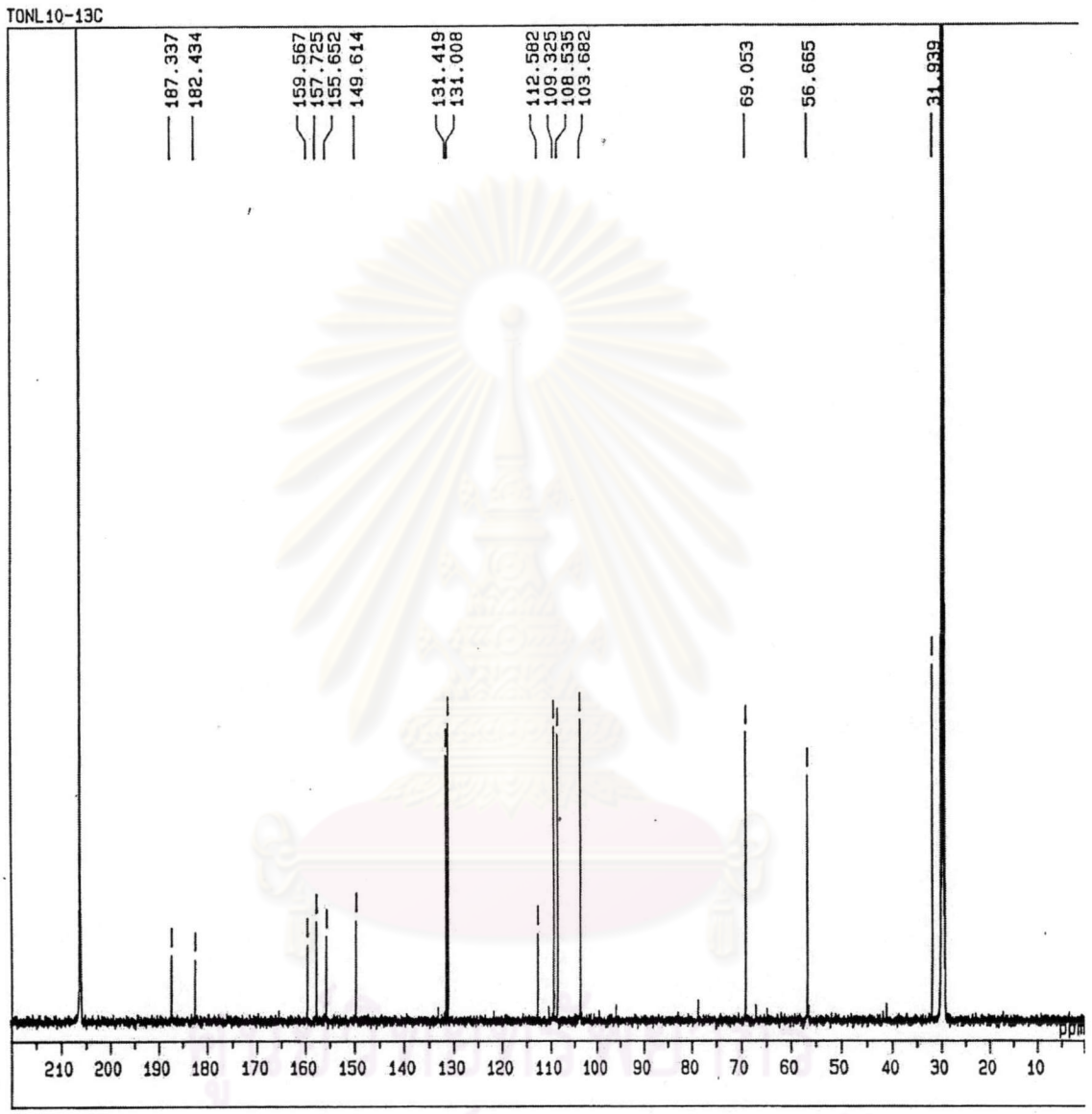


Figure 3.43 ^{13}C-NMR spectrum of compound **7**

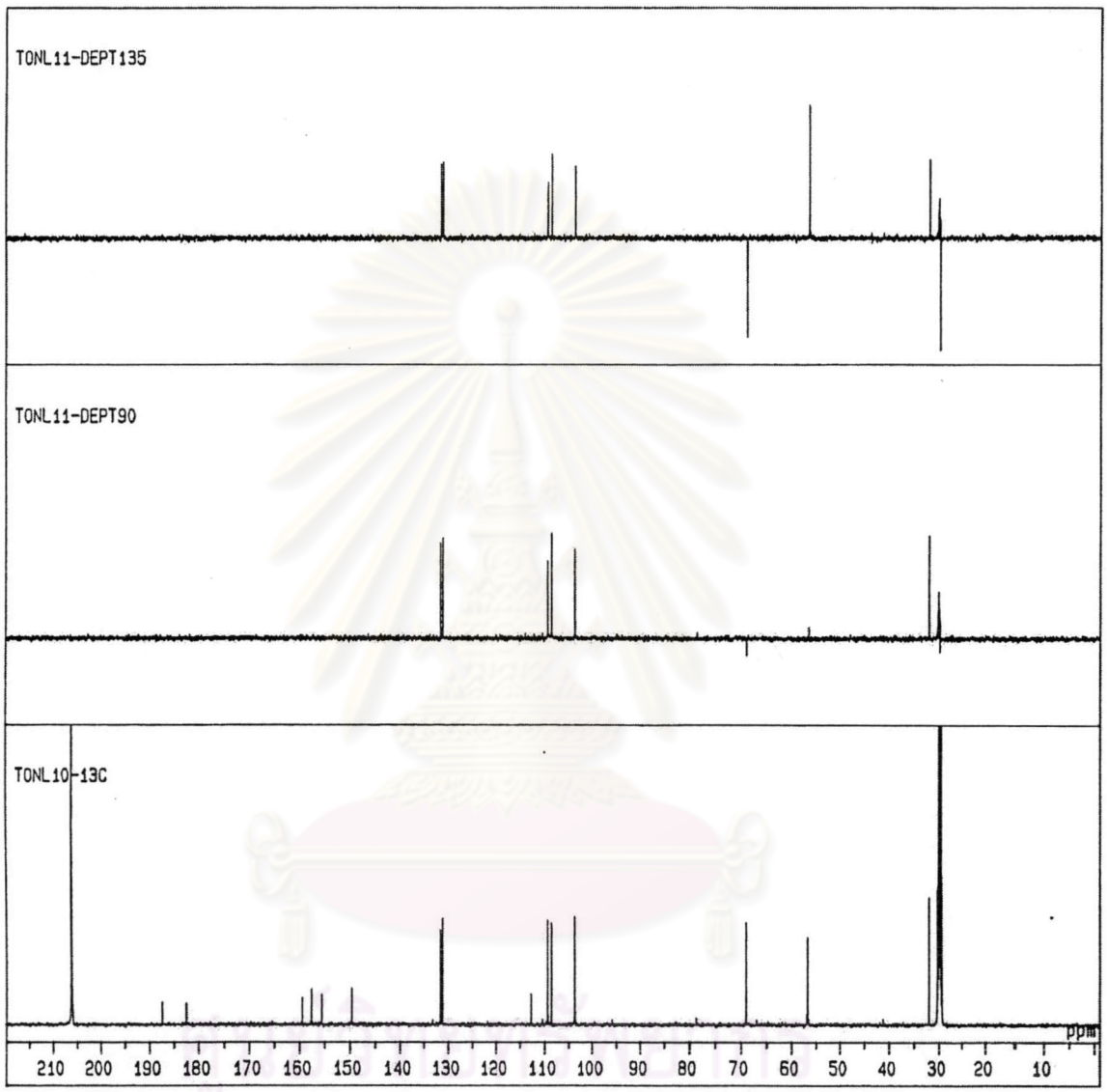


Figure 3.44 DEPT 90 and 135 spectrum of compound **7**

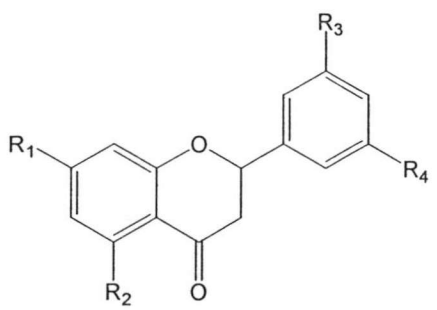
3.2.8 Compound 8

Compound **8** was obtained as white solid powder with R_f 0.12 (SiO_2, EtoAc-CH_2Cl_2, 2:8). Its melting point decomposed at 225 °C. The mass spectrum (**Figure 3.45**) displayed a molecular ion [M^+] at m/z 288 in agreement, together with fragment ion at m/z 179, 166, 153, 136 and 123. From the EI-MS, ^1H and ^{13}C-NMR data, compound 8 was determined to be $C_{15}H_{12}O_6$, indicating 10 degrees of unsaturation.

The IR spectrum (**Figure 3.46**) confirmed the presence of hydroxy group at ν_{max} 3370 cm^{-1} due to –OH stretching vibration, a carbonyl group at ν_{max} 1639 cm^{-1} due to C=O stretching vibration and exhibited double bond of benzene moiety at ν_{max} 1603, 1456 and 1449 cm^{-1}.

Its ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.47**) clearly showed signals one methylene proton δ 2.73 (1H, J = 3.1, 17.7 Hz), δ 3.24 (1H, J = 13.4, 17.7 Hz) and one methine proton at δ 5.43 (1H, J = 3.1, 13.4 Hz). From these data suggested that compound **8** had a flavanone skeleton. Two meta coupled of aromatic protons at δ 5.39 (1H, d, J = 2.1 Hz) and 5.37 (1H, d, J = 2.1 Hz) were attributable to H-6 and H-8, respectively. This spectrum also displayed two proton signals located on ring B at δ 7.02 (1H, s) and 6.86 (2H, each s). Futhermore, a hydroxyl signal due to hydrogen bond with the carbonyl group was observed at δ 12.24 ppm.

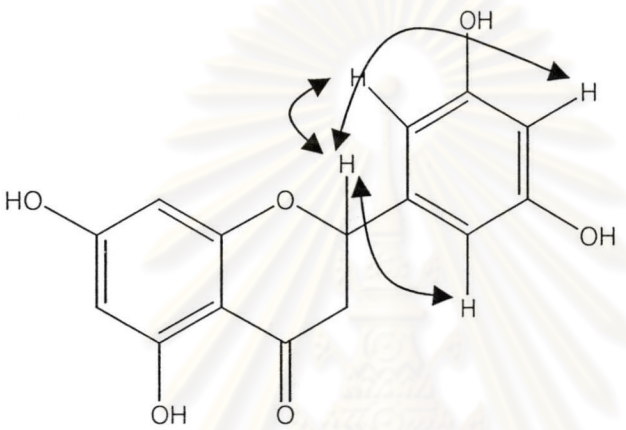
The ^{13}C-NMR spectrum (**Figure 3.48**) showed fifteen signals (flavonoid characteristic) due to two sp^3 carbons at 79.9 (C-2) and 43.5 (C-3), five oxygenated aromatic carbons at δ 165.2 (C-5), 167.3 (C-7), 164.3 (C-9), 146.3 (C-3') and 146.0 (C-5'), two quaternary aromatic carbons at δ 103.1 (C-10) and 131.4 (C-1'), five aromatic carbons bearing a proton at δ 96.7 (C-6), 95.7 (C-8), 114.6 (C-2'), 119.1 (C-4') and 115.9 (C-6') and one carbonyl carbon at δ 197.2. All of the above data suggested the possibility of flavanone skeleton as shown below.



Substituents :

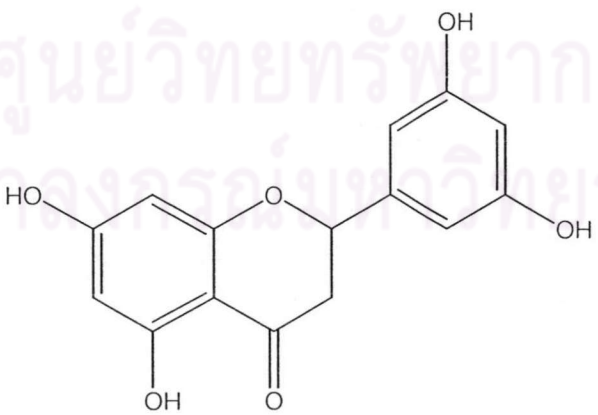
- OH (4)

The NOE DIFF experiments were used to get more additional information. Irradiation of the proton signal at δ 5.38 (H-2) ppm caused enhancement of the proton signal at δ 6.85 (H-2'), 6.86 (H-6') and 7.02 (H-4') ppm; irradiation of the proton signal at δ 7.02 ppm only resulted in NOE enhancement of H-2 signal, whereas, irradiation of the proton signal at δ 6.85 and 6.86 had effect of H-2 signal (**Figures 3.49-3.52**). The NOE DIFF correlation of compound **8** was displayed as below.



NOE DIFF correlation of compound **8**

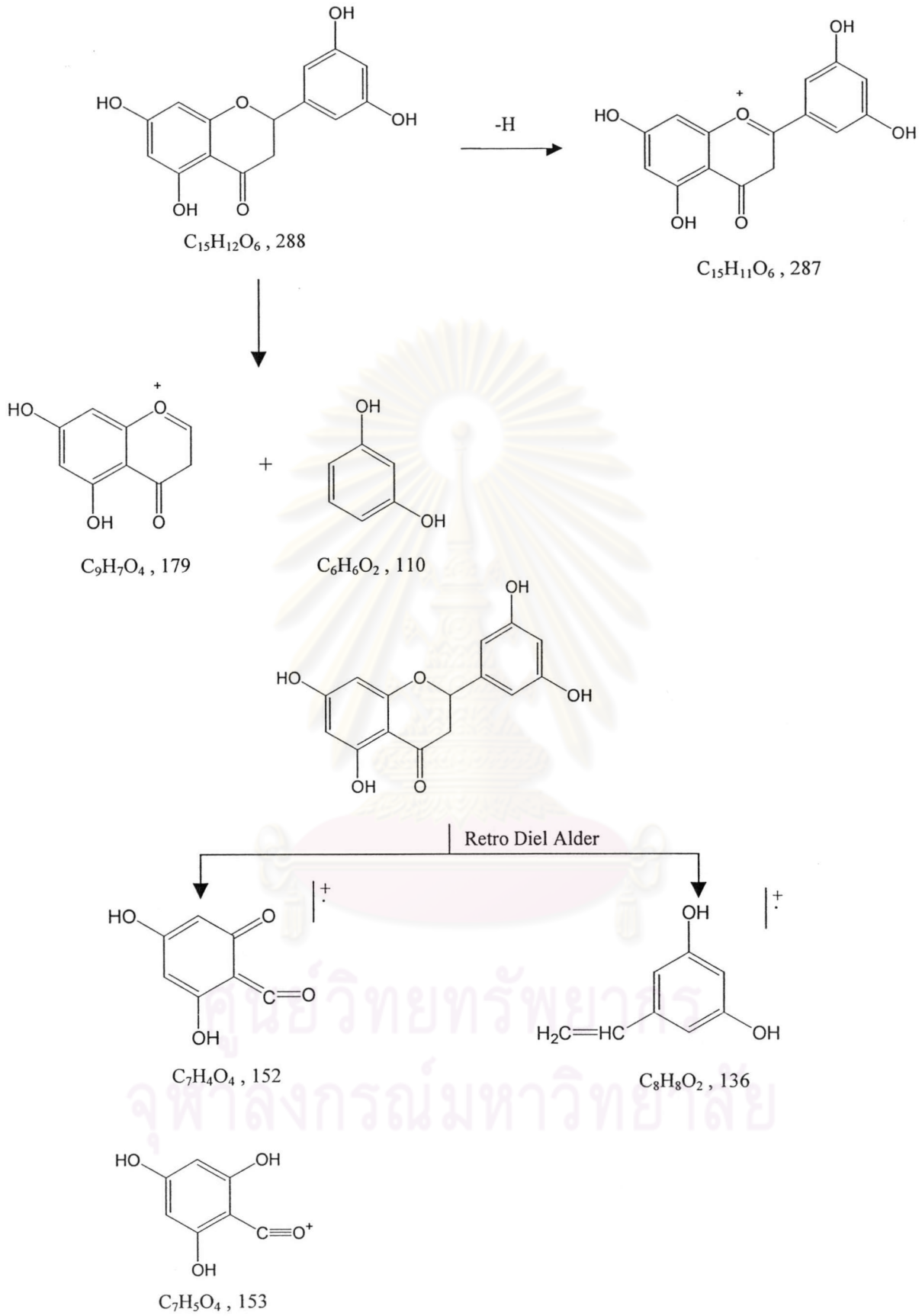
From the above data, compound **8** could be assigned as 5, 7, 3', 5'-tetrahydroxyflavanone.



Structure of compound **8**

Table 3.15 ^{1}H and ^{13}C spectral data of compound **8**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	79.9	5.38	1H, dd, J = 3, 12.8 Hz
3	43.5	3.24 2.73	1H, dd, J = 17.7, 13.4 Hz 1H, dd, J = 17.7, 3.1 Hz
4	197.2		
5	165.2		
6	96.7	5.95	1H, d, J = 2.24 Hz
7	167.3		
8	95.7	5.93	1H, d, J = 2.24 Hz
9	164.3		
10	103.1		
1'	131.4		
2'	114.6	6.85	1H, s
3'	146.3		
4'	119.1	7.02	1H, s
5'	146.0		
6'	115.9	6.86	1H, s
-OH		12.24	1H, s



Scheme 3.8 Possible mass fragmentation patterns of compound **8**

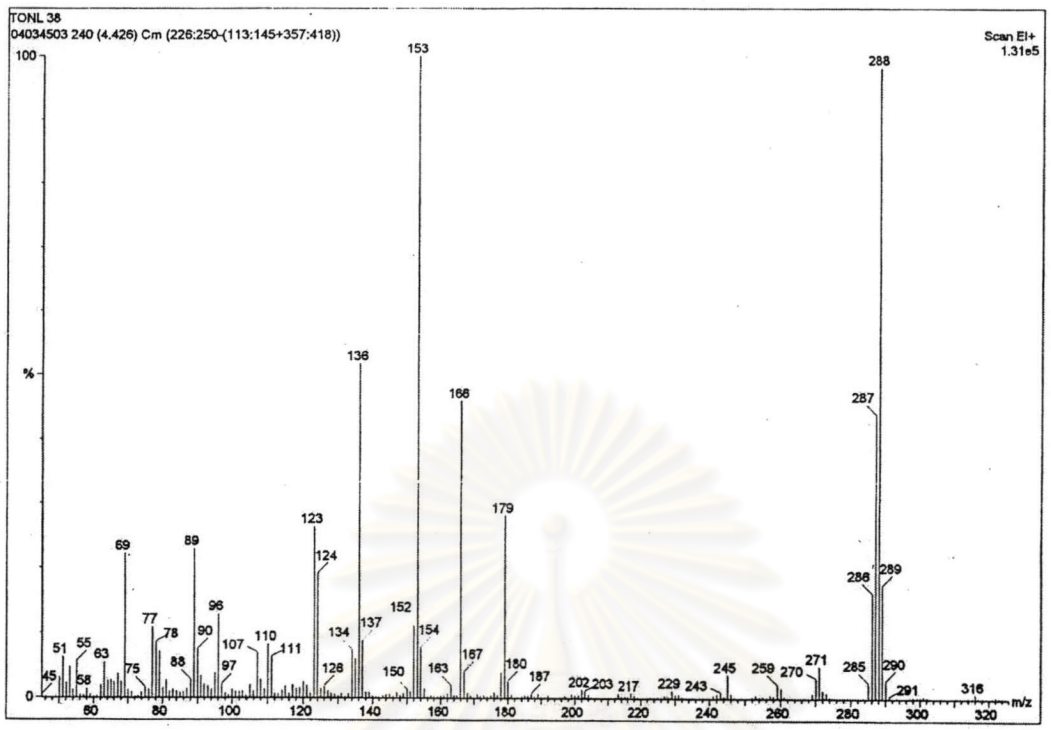


Figure 3.45 Mass spectrum of compound **8**

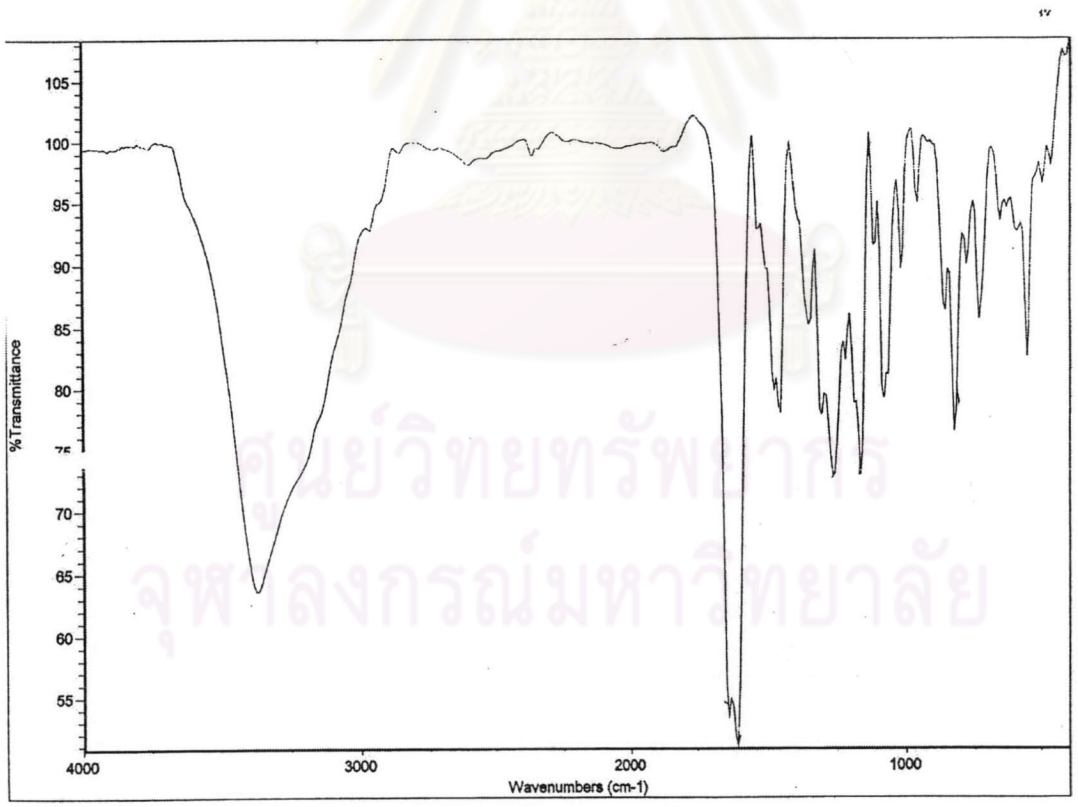


Figure 3.46 IR spectrum of compound **8**

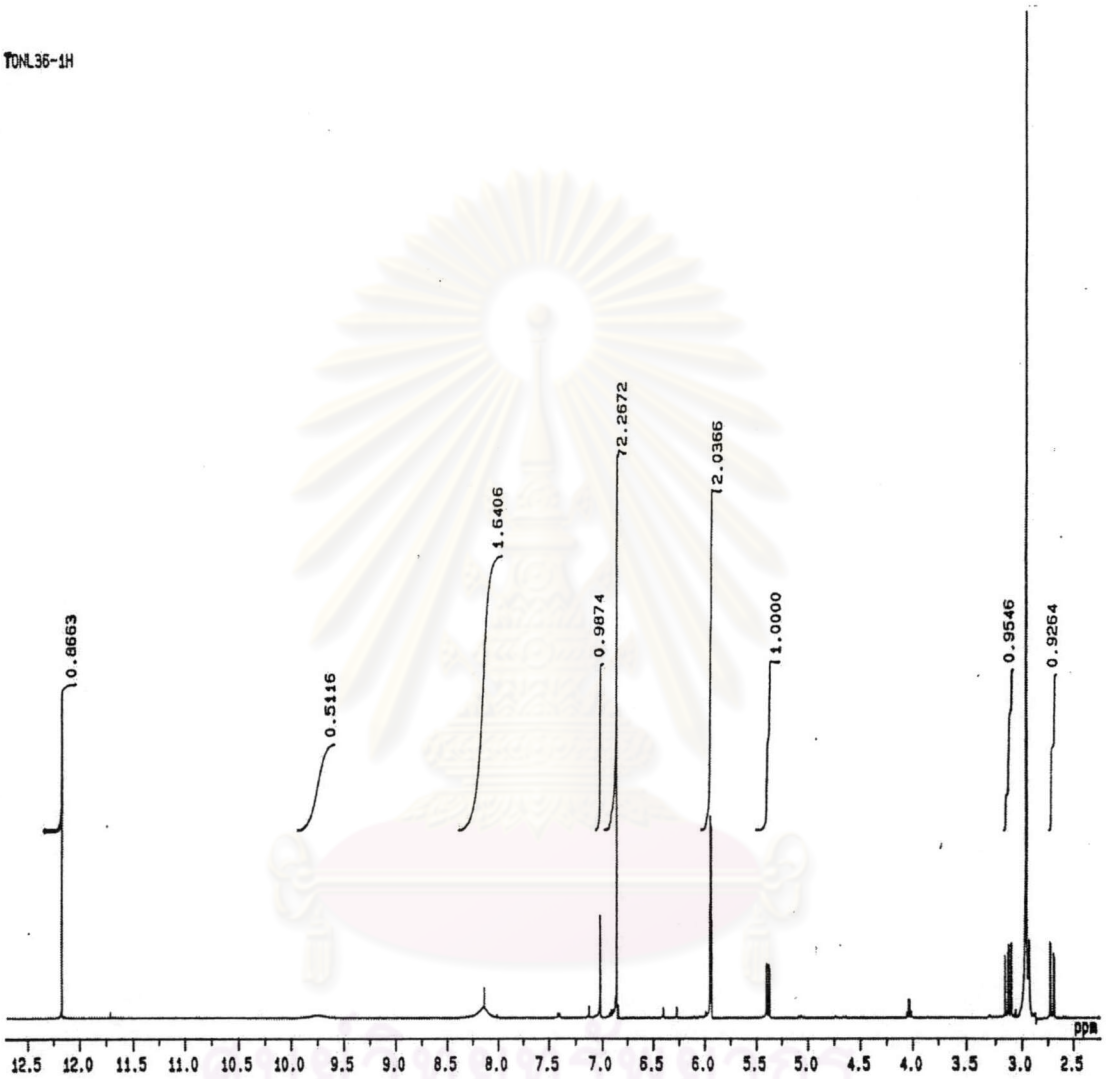


Figure 3.47 ^{1}H-NMR spectrum of compound **8**

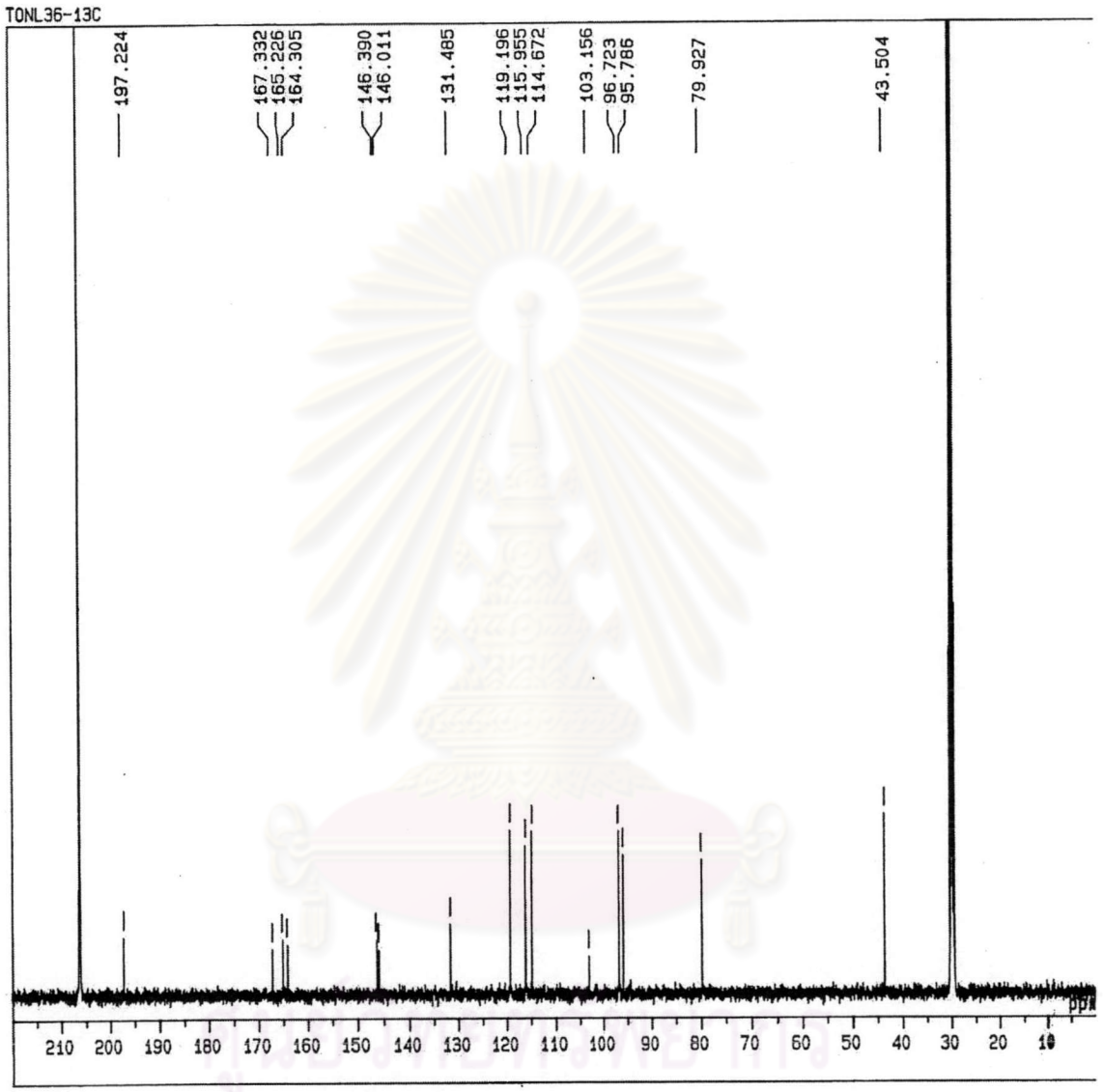


Figure 3.48 ^{13}C-NMR spectrum of compound **8**

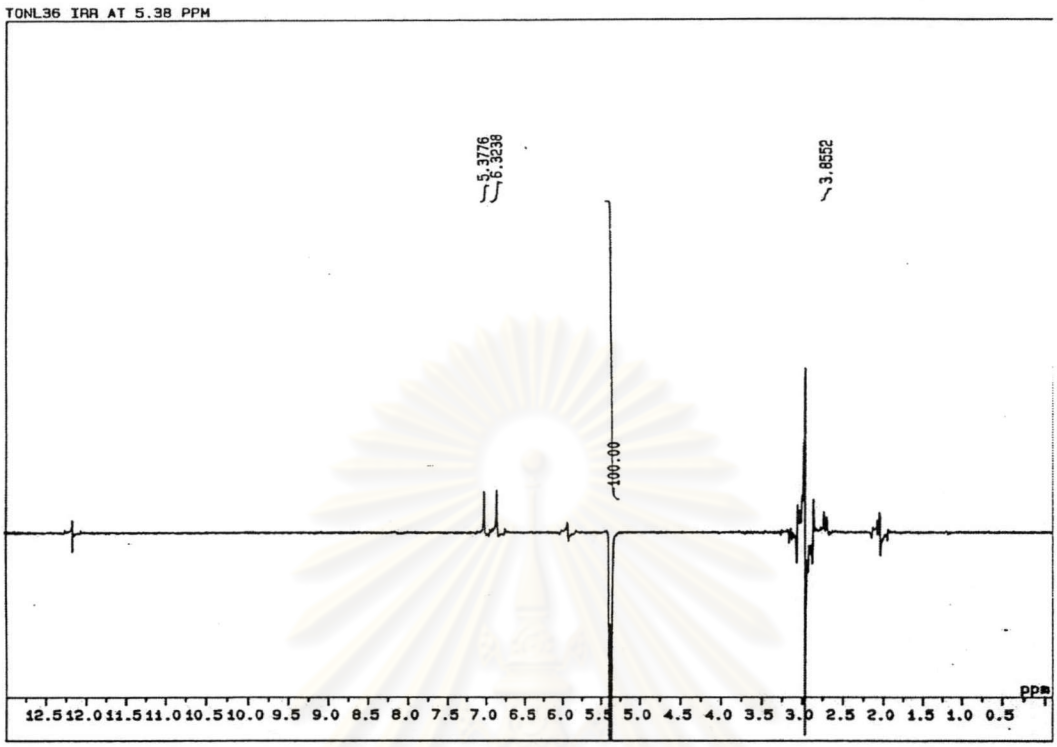


Figure 3.49 NOE DIFF of compound **8** (irradiated at δ 5.38)

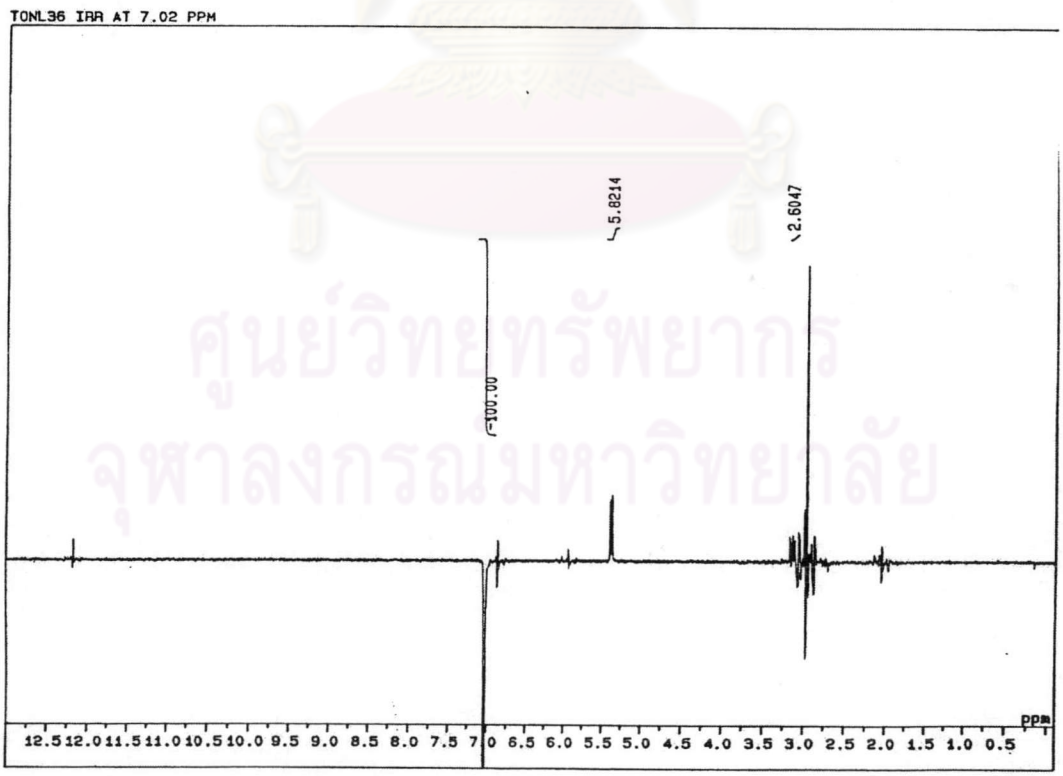


Figure 3.50 NOE DIFF of compound **8** (irradiated at δ 7.02)

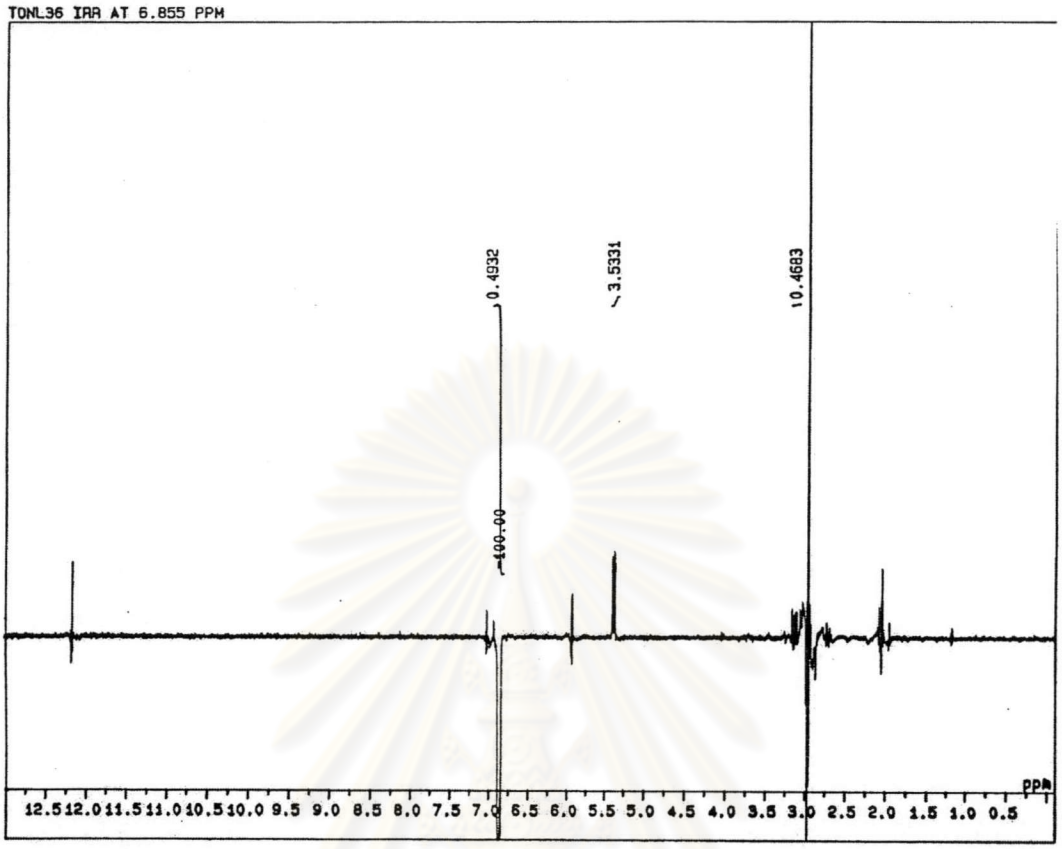


Figure 3.51 NOE DIFF of compound **8** (irradiated at δ 6.86)

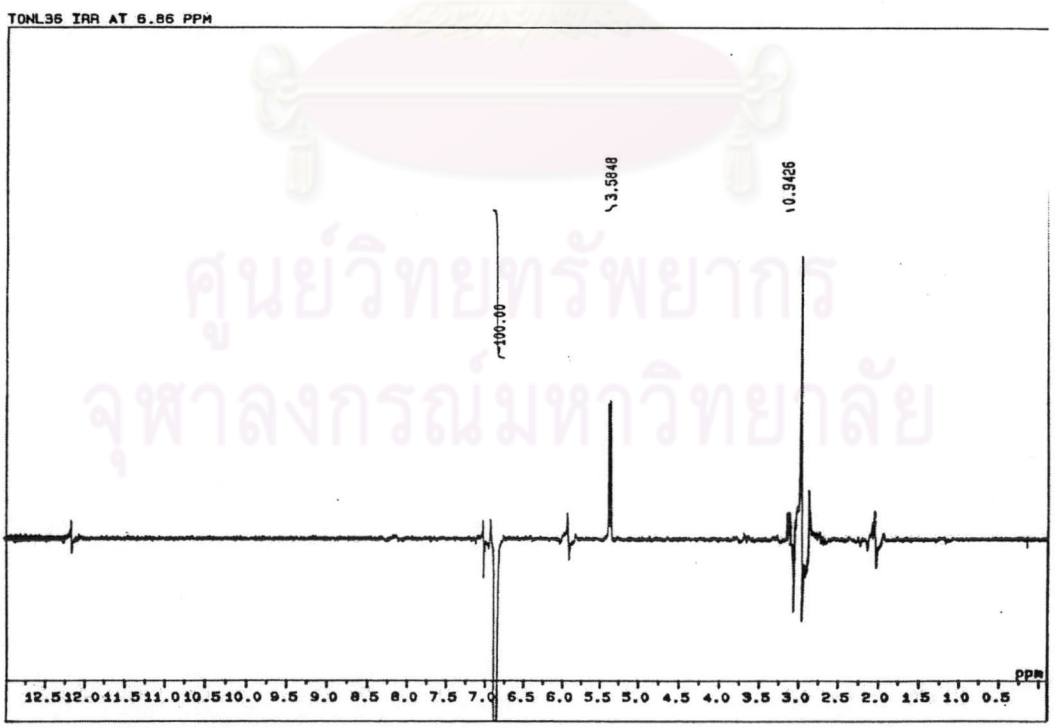


Figure 3.52 NOE DIFF of compound **8** (irradiated at δ 6.85)

3.2.9 Compound 9

White needles (2.2 g) of compound **9** were obtained from ethyl acetate crude extract and separated by column chromatography techniques, with mp. 224-228 °C and R_f value 0.66 (SiO_2, EtOAc-CH_2Cl_2, 6:4).

The IR spectrum (**Figure 3.53**) showed absorption bands at ν_{max} 3100-3400 cm^{-1} (OH stretching vibration), 1634 cm^{-1} (C=O stretching vibration) and benzene moieties stretching vibration at ν_{max} 1603, 1480 and 1311 cm^{-1}.

The molecular formula of compound **9** was determined as $C_{15}H_{12}O_5$ on the basis of EIMS, ^1H and ^{13}C-NMR data and showed also a molecular ion peak at m/z 272 [M^+] (**Figure 3.54**).

The ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.55**) of compound **9** indicated the presence of hydroxyl signal at δ 12.10 for OH-5. Two-proton meta coupled aromatic protons at δ 5.98 (2H, d, J = 2.1 Hz) were attributable to H-6 and H-8, respectively. The para disubstituted aromatic protons displayed signals at δ 7.42 (2H, d, J = 8.2 Hz) and δ 6.92 (2H, d, J = 8.2 Hz) assigned to H-2', H-6', H-3' and H-5', respectively. Signal at δ 3.24 (1H, dd, J = 17, 12 Hz) and δ 2.73 (1H, dd, J = 17, 3 Hz) were assigned for methylene proton (two protons) of H-3. The one-proton doublet-doublet at δ 5.43 (1H, dd, J = 12.8, 3 Hz) was assigned to H-2.

The ^{13}C-NMR spectrum (**Figure 3.56**) indicated the presence of 15 distinct carbon signals of the flavanone moiety, including one carbonyl carbon (δ 197.2, C-4) and two alicyclic carbons (δ 79.8, C-2 and δ 43.4, C-3). The aromatic region contained a total of 12 signals. They were six tertiary carbons (δ 95.7, C-8; δ 96.7, C-6; δ 116.1, C-3', C-5'; δ 128.9, C-2', C-6') and six quaternary carbons (δ 165.2, 167.2, 164.3, 103.1, 130.7 and 158.6 for C-5, C-7, C-9, C-10, C-1' and C-4', respectively). The complete ^1H and ^{13}C-NMR data assignment of compound **9** were shown in **Table 3.16**.

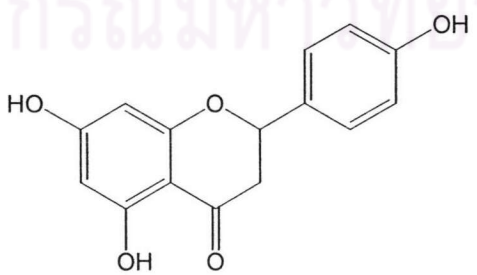
Table 3.16 ^{1}H and ^{13}C spectral data of compound **9**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	79.8	5.43	1H, dd, J = 12.8, 3 Hz
3	43.4	3.24, 2.73	1H, dd, J = 17, 12 Hz ; 1H, dd, J = 17, 3 Hz
4	197.2		
5	165.2		
6	96.7	5.98	1H, d, J = 2.1 Hz
7	167.2		
8	95.7	5.98	1H, d, J = 2.1 Hz
9	164.3		
10	103.1		
1'	130.7		
2'	128.9	7.42	1H, d, J = 8.2 Hz
3'	116.1	6.92	1H, d, J = 8.2 Hz
4'	158.6		
5'	116.1	6.92	1H, d, J = 8.2 Hz
6'	128.9	7.42	1H, d, J = 8.2 Hz
-OH		12.17	1H, s

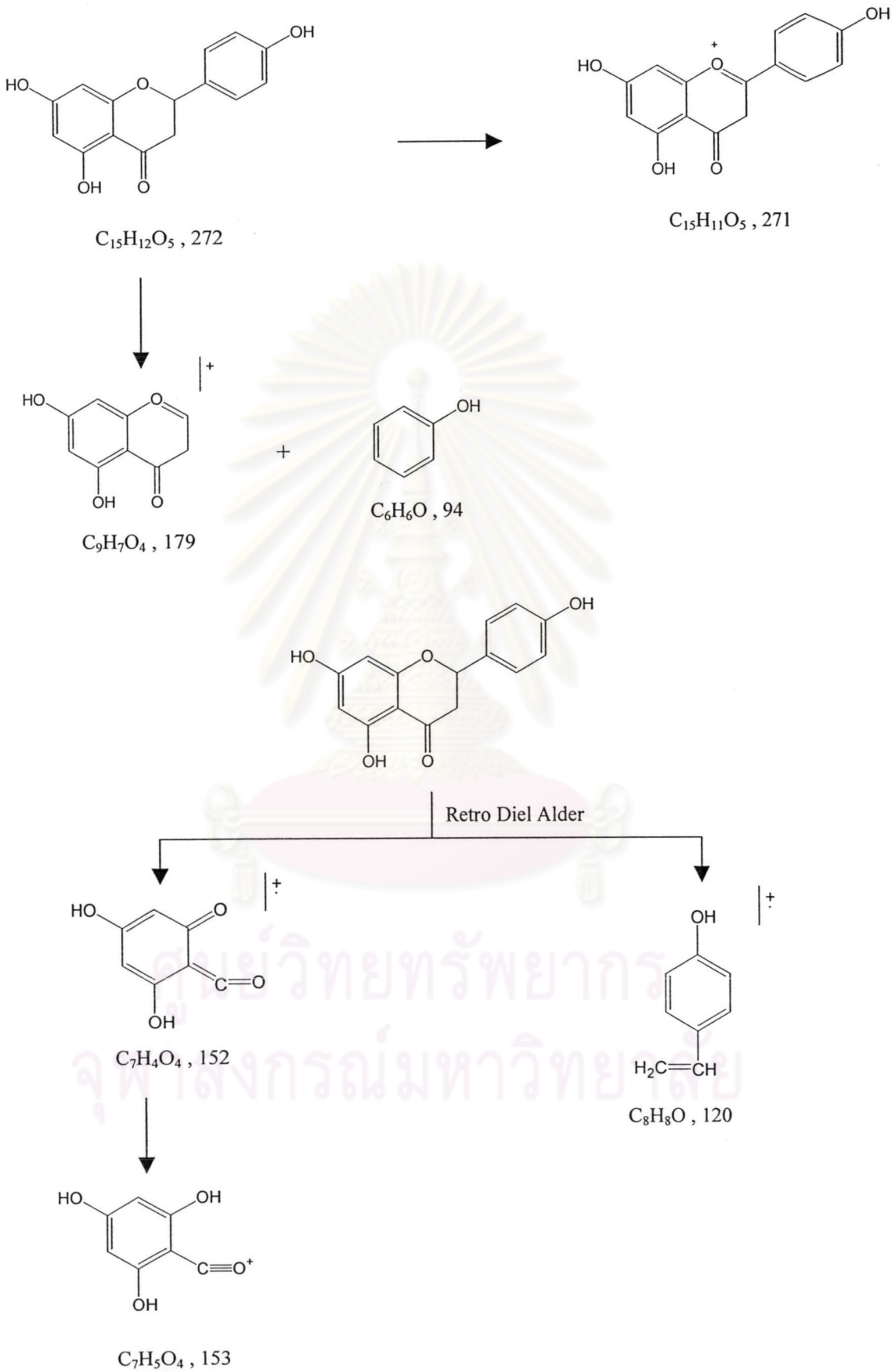
Table 3.17 ^{13}C NMR data of compound **9** compared with Naringenin.[21]

Position	Compound **9**	Naringenin
2	79.8	78.4
3	43.4	42.0
4	197.2	196.2
5	165.2	163.6
6	96.7	95.9
7	167.2	166.7
8	95.7	95.0
9	164.3	162.9
10	103.1	101.8
1'	130.7	128.9
2'	128.9	128.2
3'	116.1	115.2
4'	158.6	157.8
5'	116.1	115.2
6'	128.9	128.2

From the spectral data, and comparison of ^{13}C-NMR signals of compound **9** with reported data of naringenin. Thus, this compound was established as 5,7,4'-trihydroxyflavanone or naringenin.



The structure of compound **9** (naringenin)



Scheme 3.9 Possible mass fragmentation patterns of compound **9**

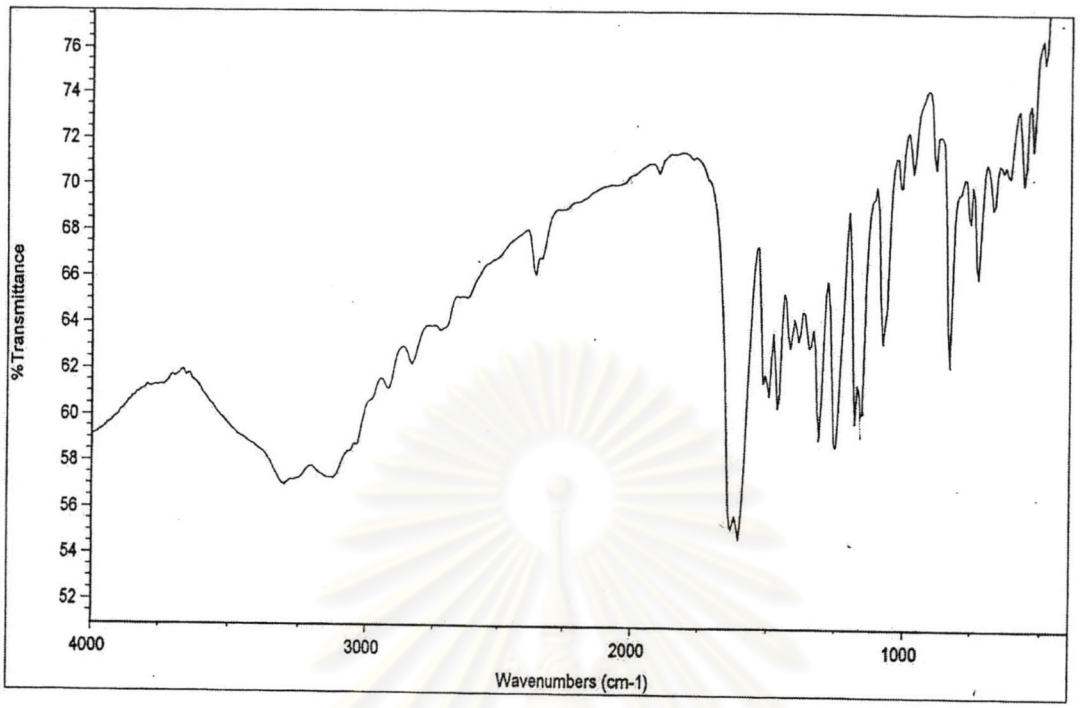


Figure 3.53 IR spectrum of compound **9**

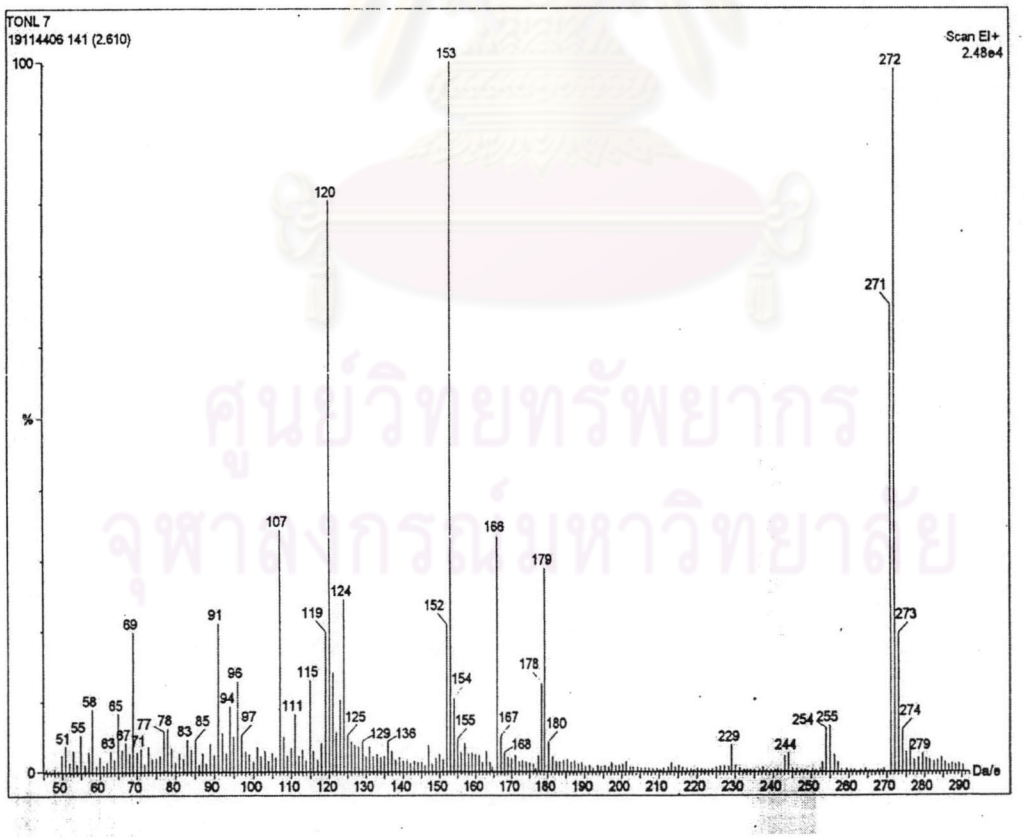


Figure 3.54 Mass spectrum of compound **9**

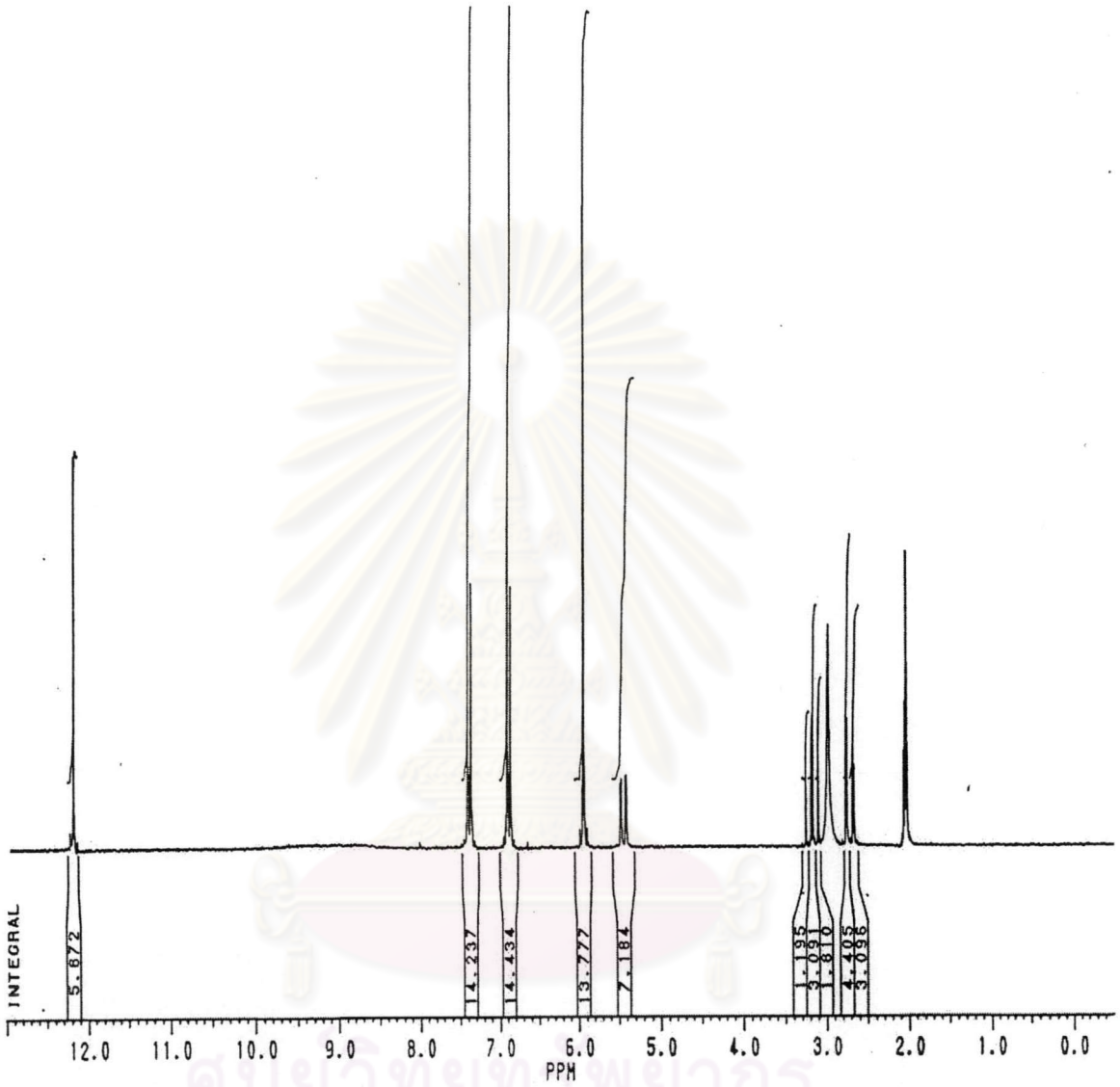


Figure 3.55 ^{1}H-NMR spectrum of compound **9**

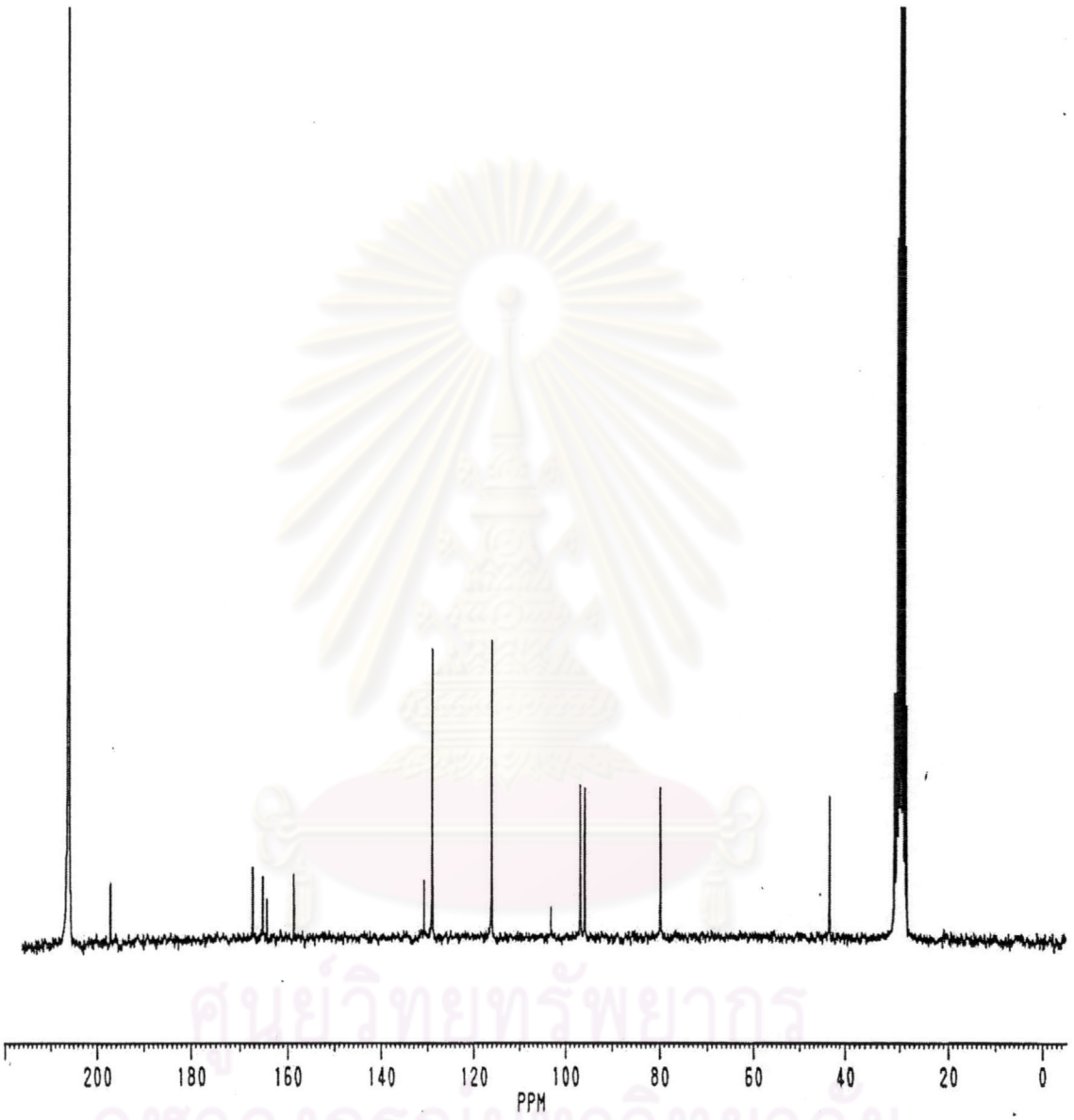


Figure 3.56 ^{13}C-NMR spectrum of compound **9**

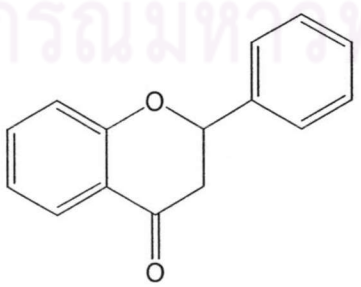
3.2.10 Compound 10

Compound **10** was isolated as a white solid by open column chromatography on a silica gel and decomposed at 200°C. Its R_f value was 0.34 (SiO_2, EtOAc-CH_2Cl_2, 2:8). The mass spectrum (**Figure 3.57**) gave a molecular ion peak [M^+] at m/z 256 in agreement with the empirical formula $C_{15}H_{12}O_4$, corresponding to 10 degrees of unsaturation.

The IR spectrum (**Figure 3.58**) showed absorption band for hydroxyl (ν_{max} 3308 cm^{-1}), carbonyl (ν_{max} 1654 cm^{-1}) functionalities and double bond of aromatic (ν_{max} 1613, 1501 cm^{-1}).

The ^{1}H-NMR spectrum data (acetone-d_6, 500 MHz) (**Figure 3.59**) indicated the presence of one methylene (δ 2.73 and 3.24) and one oxygen-substituted methine group at δ 5.43. The remaining signals in the ^{1}H-NMR spectrum indicated the seven aromatic protons at δ 7.70 (1H, d, J = 8.6 Hz), 6.57 1H, dd, J = 2.1, 8.6 Hz), 6.40 (1H, d , J = 2.2 Hz) and signal for p-disubstituted aromatic protons at δ 7.40 (2H, d, J = 8.5 Hz) and 6.90 (2H, d, J = 8.5 Hz).

The ^{13} C-NMR spectrum (**Figure 3.60**) indicated the presence of fifteen distinct carbon resonance of the flavanone skeleton, including one carbonyl carbon at δ 190.8 and two alicyclic carbons at δ 80.4 (C-2) and 44.6 (C-3). The aromatic region contained a total of 12 resonances. These were seven tertiary carbons at δ 129.4 (C-5), 111.1 (C-6), 103.6 (C-8), 128.9 (C-2' and C-6') and 116.0 (C-3' and C-5') and five quaternary carbons at δ 165.2 (C-7), 164.2 (C-9), 115.0 (C-10), 131.0 (C-1') and 158.3 (C-4'). The complete assignment for protons and carbons of compound **10** was shown in **Table 3.18**.



Flavanone skeleton

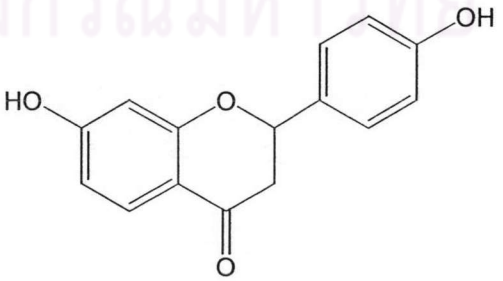
Table 3.18 ^{1}H and ^{13}C spectral data of compound **10**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	80.5	5.43	1H, dd, J = 12.9, 3 Hz
3	44.6	3.24, 2.73	1H, dd, J = 17, 12 Hz ; 1H, dd, J = 17, 3 Hz
4	190.8		
5	129.4	7.70	1H, d, J = 8.6 Hz
6	111.1	6.57	1H, dd, J = 2.1, 8.6 Hz
7	165.2		
8	103.6	6.40	1H, d, J = 2.2 Hz
9	164.2		
10	115.0		
1'	131.0		
2'	128.9	7.40	1H, d, J = 8.7 Hz
3'	116.0	6.90	1H, d, J = 8.2 Hz
4'	158.3		
5'	116.0	6.90	1H, d, J = 8.2 Hz
6'	128.9	7.40	1H, d, J = 8.7 Hz

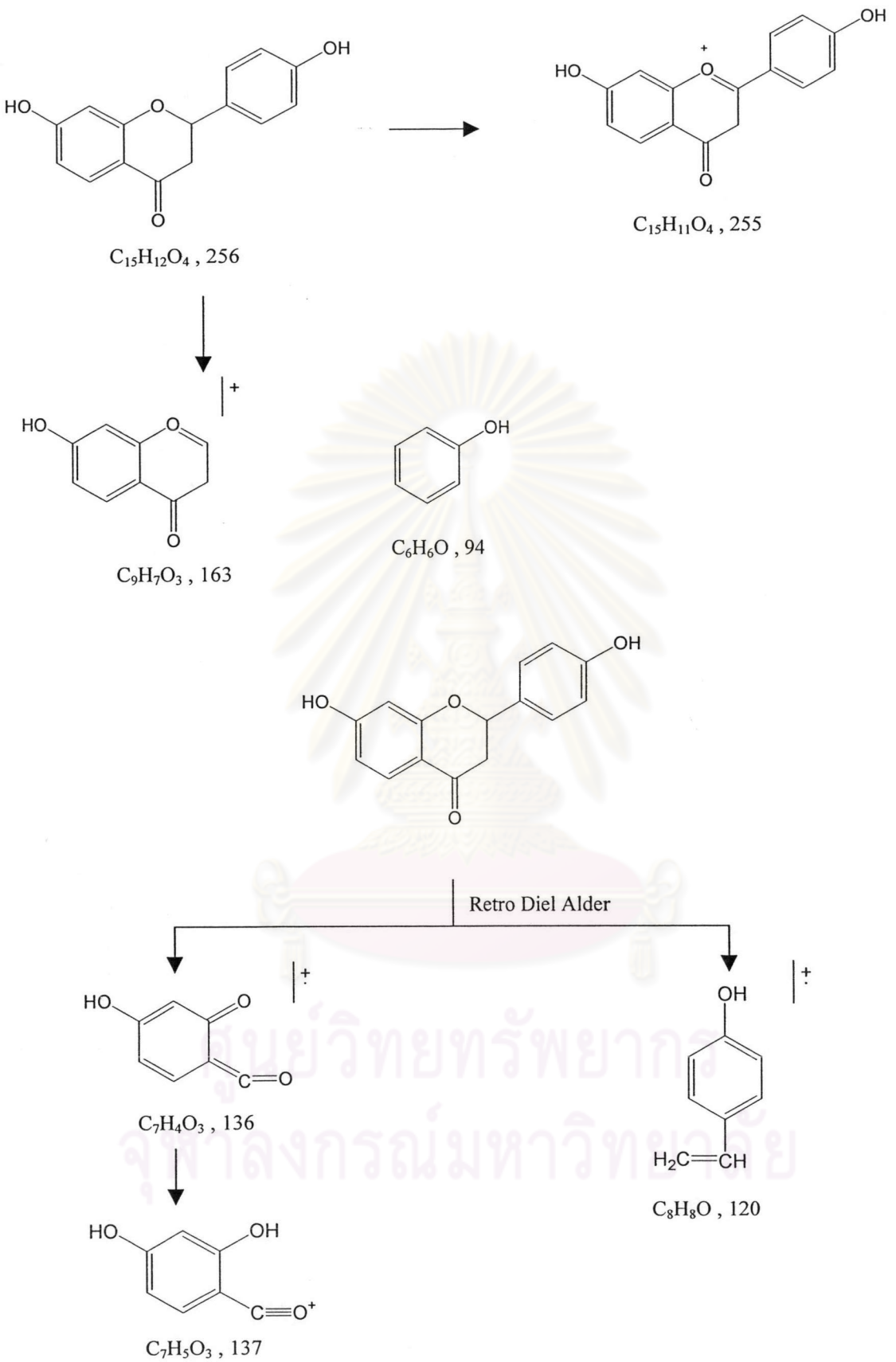
Table 3.19 ^{13}C NMR data of compound **10** compared with Liquiritigenin.[22]

Position	Compound **10**	Liquiritigenin
2	80.5	80.4
3	44.6	44.5
4	190.8	190.8
5	129.4	129.4
6	111.1	111.2
7	165.2	165.3
8	103.6	103.5
9	164.2	164.5
10	115.0	115.1
1'	131.0	131.1
2'	128.9	128.8
3'	116.0	116.3
4'	158.3	158.5
5'	116.0	116.3
6'	128.9	128.8

On the basis of spectroscopic observation and comparison of ^{13}C-NMR spectrum of compound **10** with published data of liquiritigenin, this compound was established as 7,4'-dihydroxyflavanone or liquiritigenin.



The structure of compound **10** (liquiritigenin)



Scheme 3.10 Possible mass fragmentation patterns of compound **10**

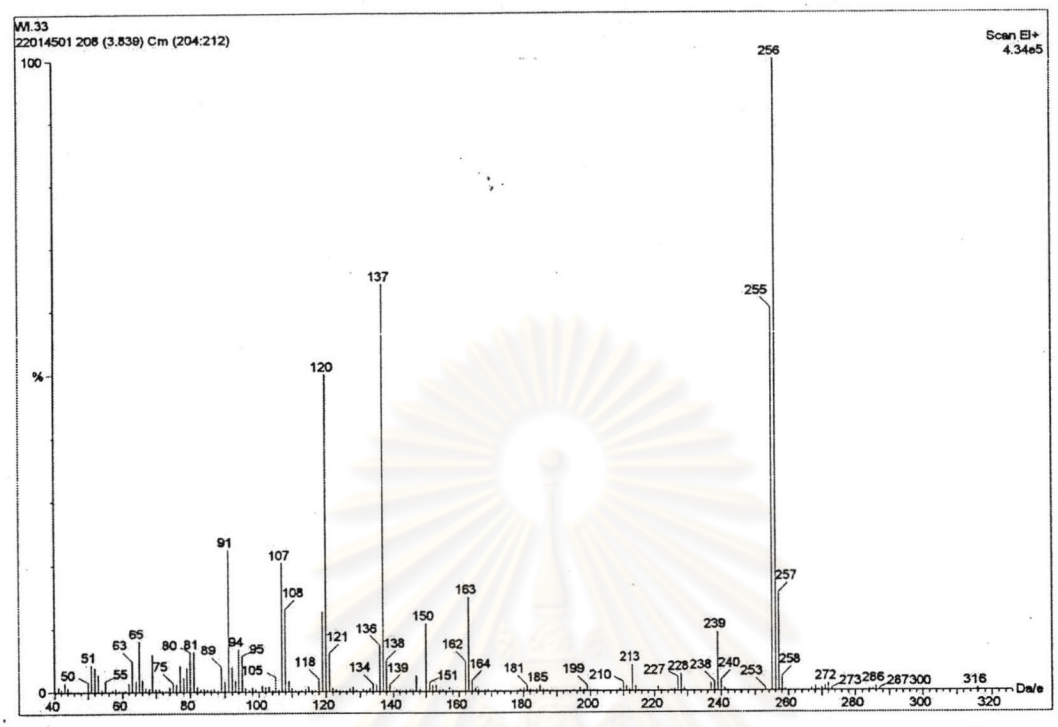


Figure 3.59 Mass spectrum of compound **10**

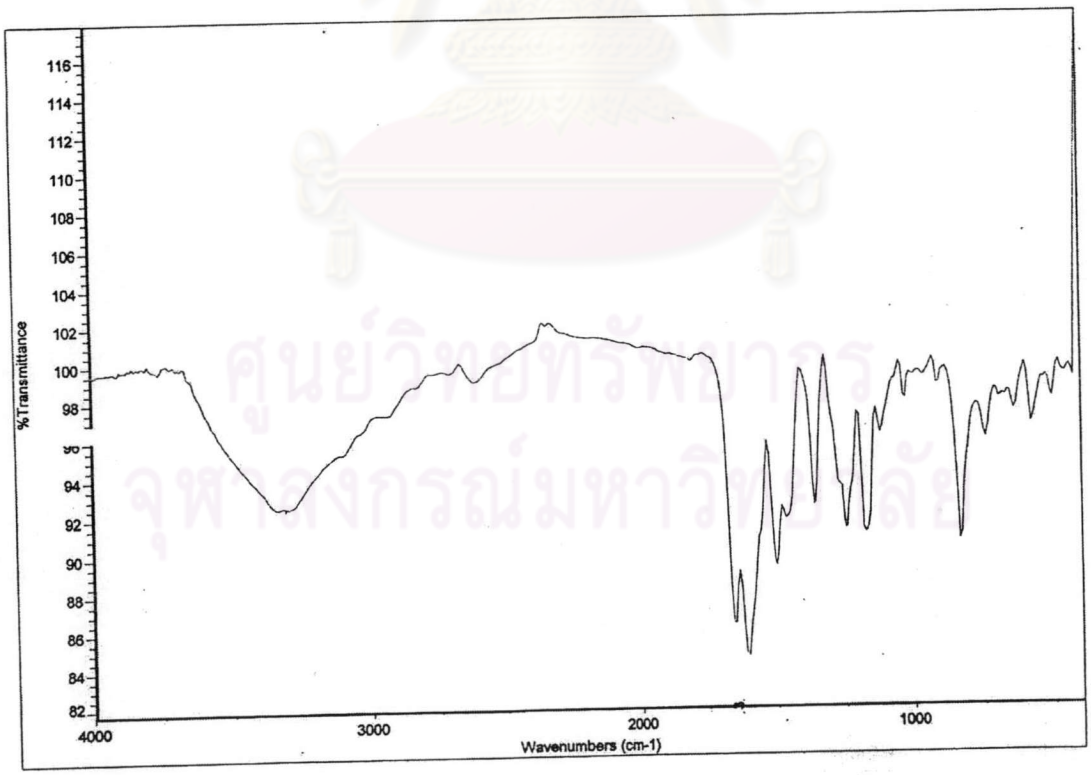


Figure 3.60 IR spectrum of compound **10**

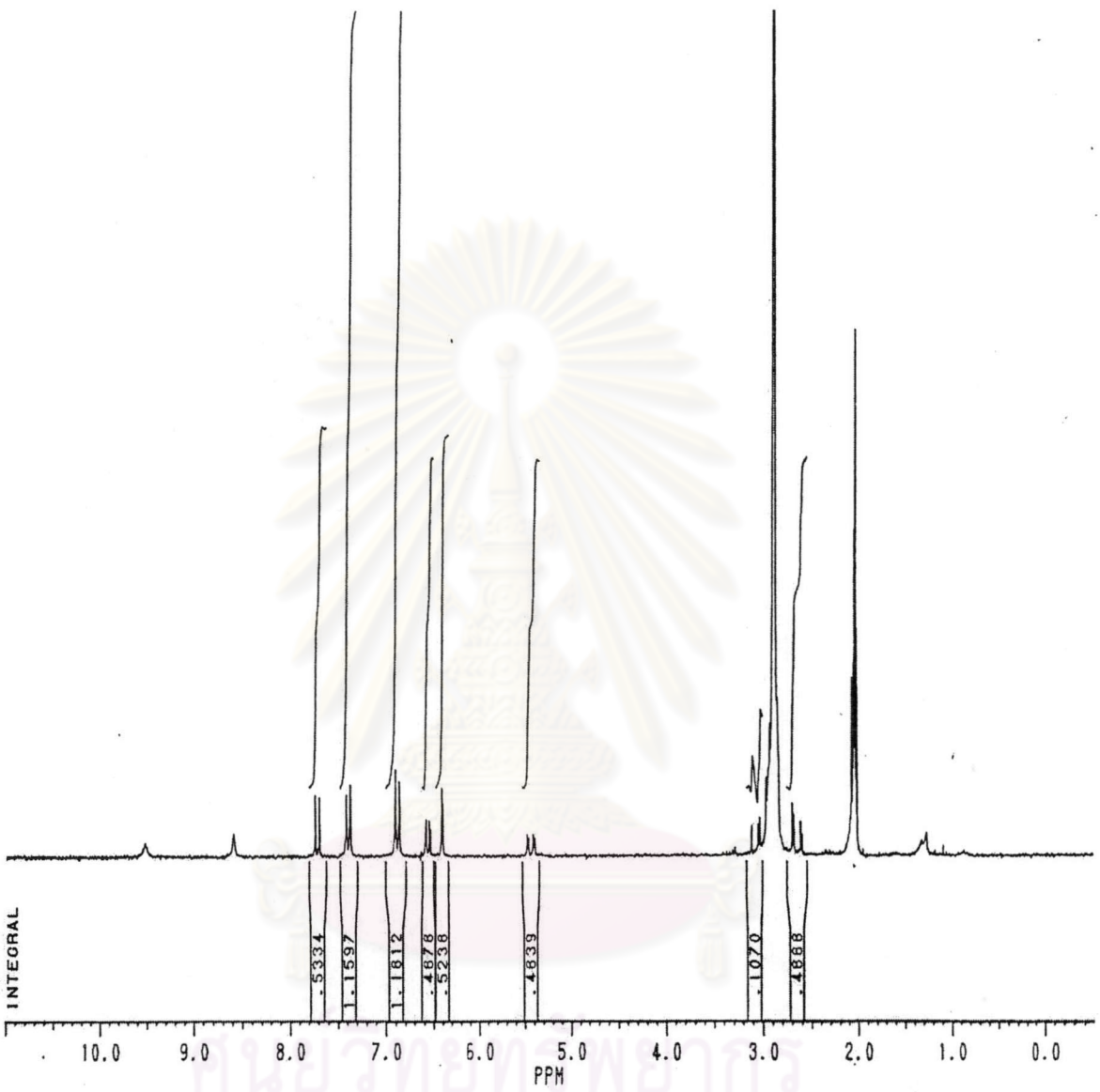


Figure 3.59 ^{1}H-NMR spectrum of compound **10**

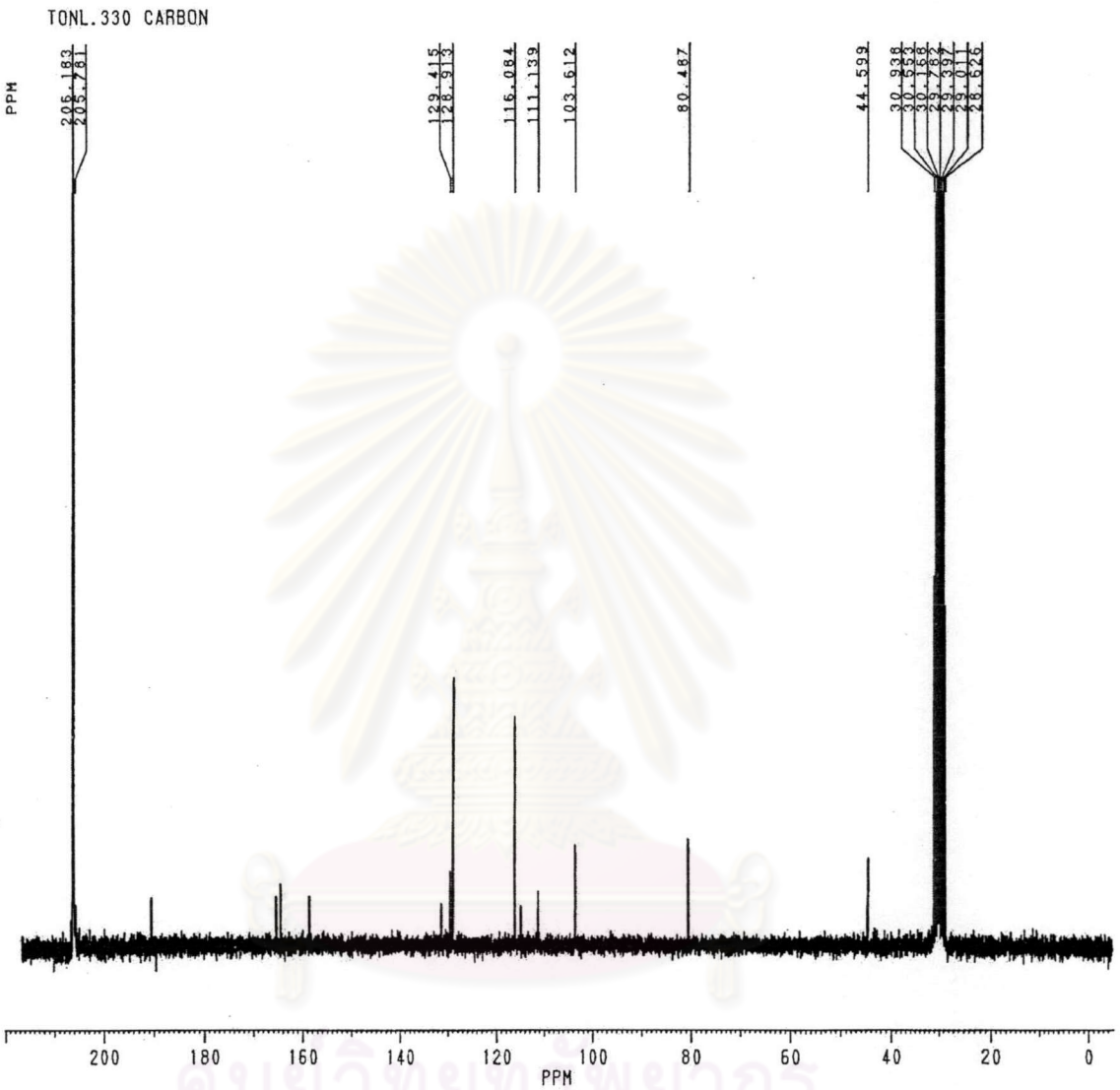


Figure 3.60 ^{13}C-NMR spectrum of compound **10**

3.2.11 Compound 11

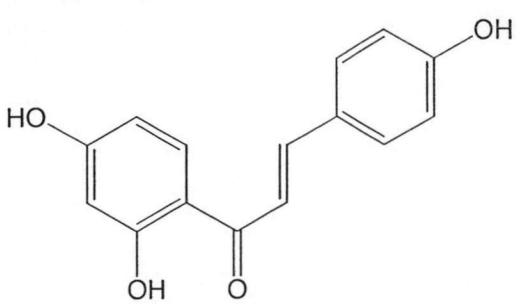
Compound **11**, yellow needles, mp. 152-154 °C with R_f 0.6 (SiO_2, EtOAc-CH_2Cl_2, 6:4), showed a molecular ion at m/z 256 together with other fragment ions at m/z 163, 137 and 120 in the mass spectrum (**Figure3.61**). Compound **11** can be dissolved in acetone, methanol and ethyl acetate but not in dichloromethane and hexanes.

The IR spectrum (**Figure 3.62**) exhibited the presence of free hydroxy (ν_{max} 3380 cm^{-1}), conjugated carbonyl (ν_{max} 1634 cm^{-1}) and olefine (ν_{max} 1567, 1516 cm^{-1}) functionalities. The molecular formula of $C_{15}H_{12}O_4$ was established by MS, ^1H and ^{13}C-NMR data.

Its ^1H-NMR spectrum (acetone-d_6, 500 MHz) (**Figure 3.63**) showed, in the sp^2 region, the signals of nine protons consisting of a pair of ABX type signals [δ 6.35 (1H, d, J = 2.4 Hz), 6.45 (1H, dd, J = 2.4, 8.8 Hz), 8.12 (1H, d, J = 8.8 Hz)], a pair of A_2B_2 type signals [δ 7.72 (2H, d, J = 8.7 Hz), 6.90 (2H, d, J = 8.7 Hz)] and two proton signals [δ 7.75, s]. Besides the above signals, a hydroxyl signal due to hydrogen bond to carbonyl group was observed near δ 13.6 ppm.

The ^{13}C-NMR spectrum (**Figure 3.64**), fifteen carbon signals were observed due to three oxygenated carbons at δ 165.5 (C-2'), 167.5 (C-4') and 160.9 (C-4), two quaternary carbons at δ 114.1 (C-1') and 127.5 (C-1), nine carbons bearing an aromatic proton at δ 103.6 (C-3'), 108.6 (C-5'), 133.2 (C-6'), 131.7 (2C, C-2, C-6) and 116.7 (2C, C-3, C-5), α and β carbons conjugated to carbonyl carbon (118.2-Cα , 145.1-Cβ) and one carbonyl carbon at δ 192.8 (C=O).

From the above data, compound **11** could be chalcone skeleton. This structure was confirmed by comparison of carbon data of this compound with previously reported data of isoliquiritigenin. Thus, compound **11** was found to be 2', 4', 4-trihydroxychalcone or isoliquiritigenin as shown below:



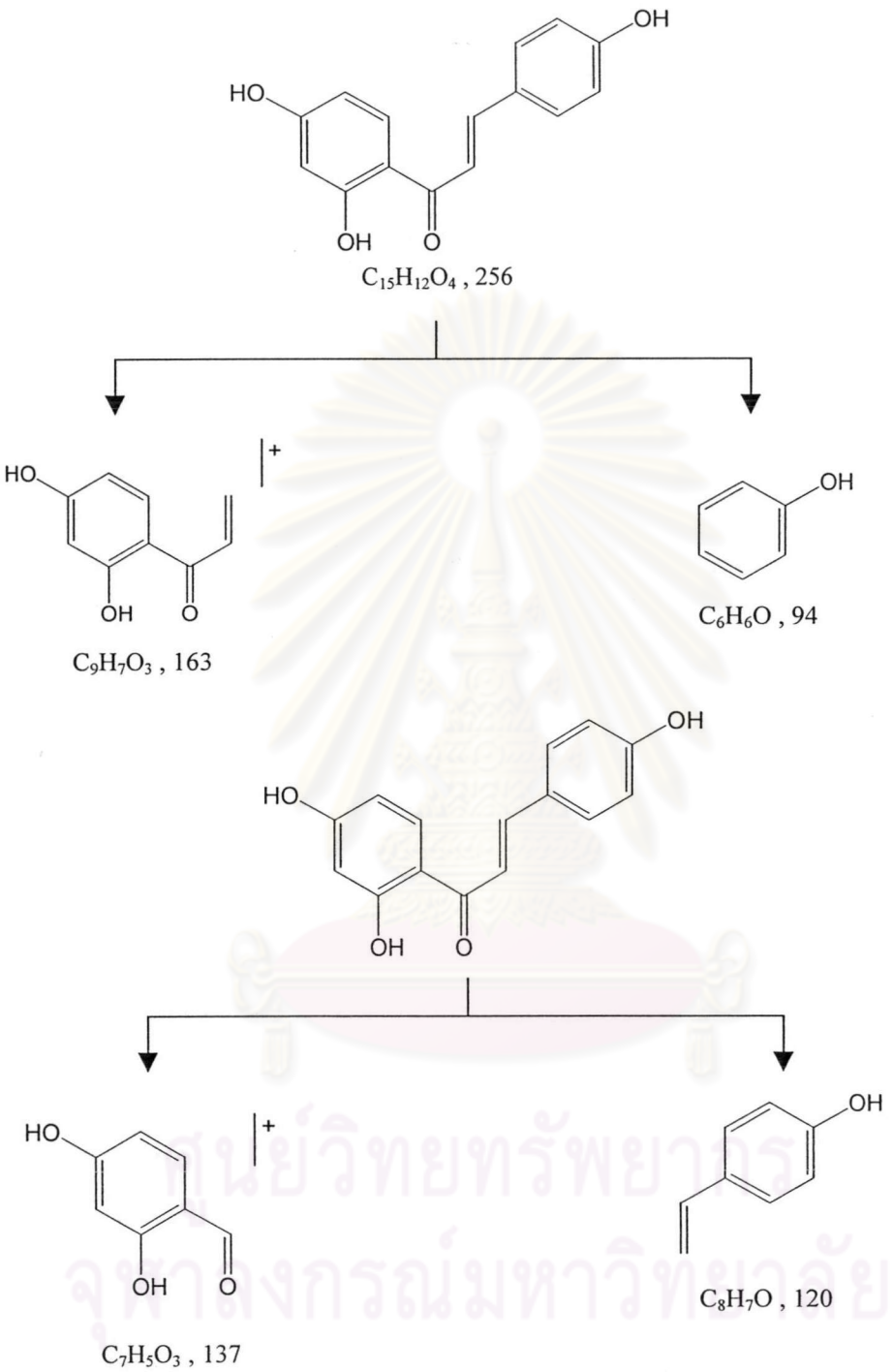
The structure of compound **11** (isoliquiritigenin)

Table 3.20 ^{1}H and ^{13}C spectral data of compound **11**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
1	127.5		
2	131.7	7.72	1H, d, J = 8.7 Hz
3	116.7	6.90	1H, d, J = 8.7 Hz
4	160.9		
5	116.7	6.90	1H, d, J = 8.7 Hz
6	131.7	7.72	1H, d, J = 8.7 Hz
1'	114.1		
2'	165.5		
3'	103.6	6.35	1H, d, J = 2.4
4'	167.5		
5'	108.6	6.45	1H, dd, J = 2.4, 8.8 Hz
6'	133.2	8.12	1H, d, J = 8.8 Hz
α	118.2	7.75	1H, s
β	145.1	7.75	1H, s
-OH		13.63	1H, s

Table 3.21 ^{13}C NMR data of compound **11** compared with Isoliquiritigenin.[22]

Position	Compound **11**	isoliquiritigenin
1	127.5	127.3
2	131.7	131.5
3	116.7	116.7
4	160.9	160.8
5	116.7	116.7
6	131.7	131.5
1'	114.1	114.3
2'	165.5	165.5
3'	103.6	103.7
4'	167.5	167.4
5'	108.6	108.6
6'	133.2	133.0
α	118.2	118.1
β	145.1	145.0



Scheme 3.11 Possible mass fragmentation patterns of compound **11**

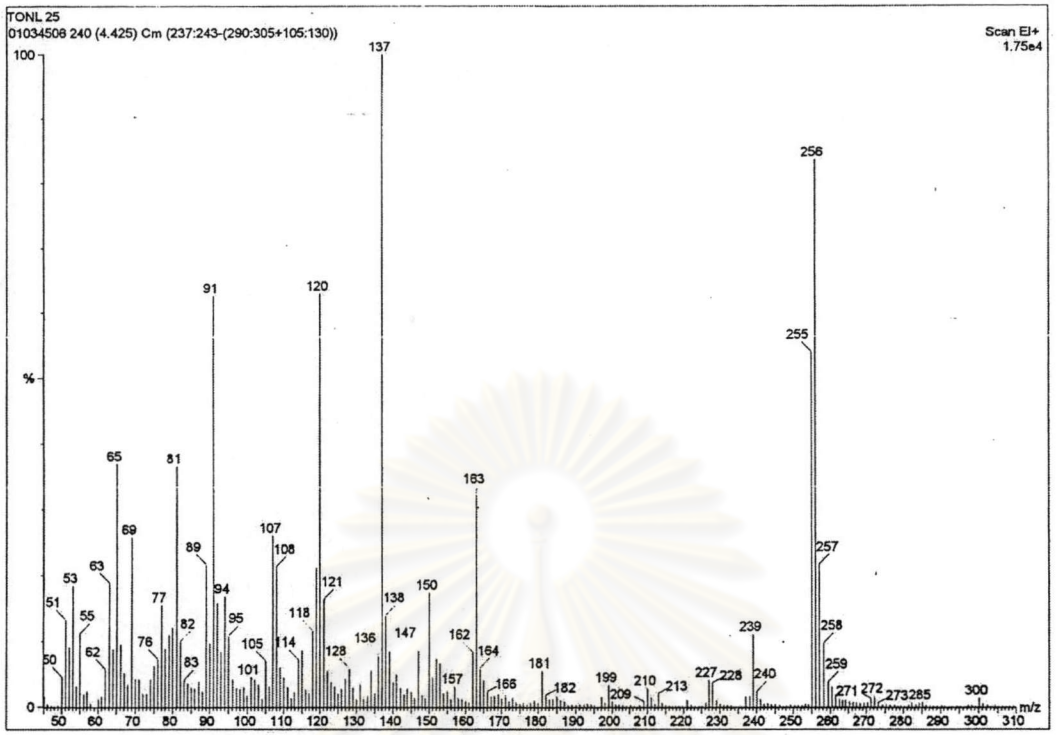


Figure 3.61 Mass spectrum of compound **11**

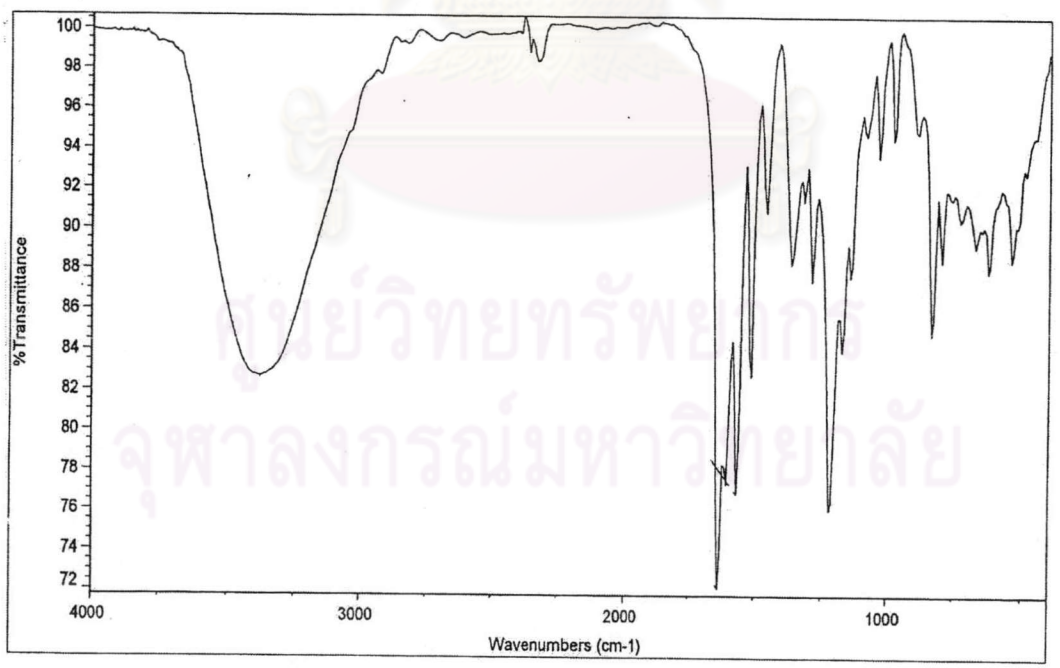


Figure 3.62 IR spectrum of compound **11**

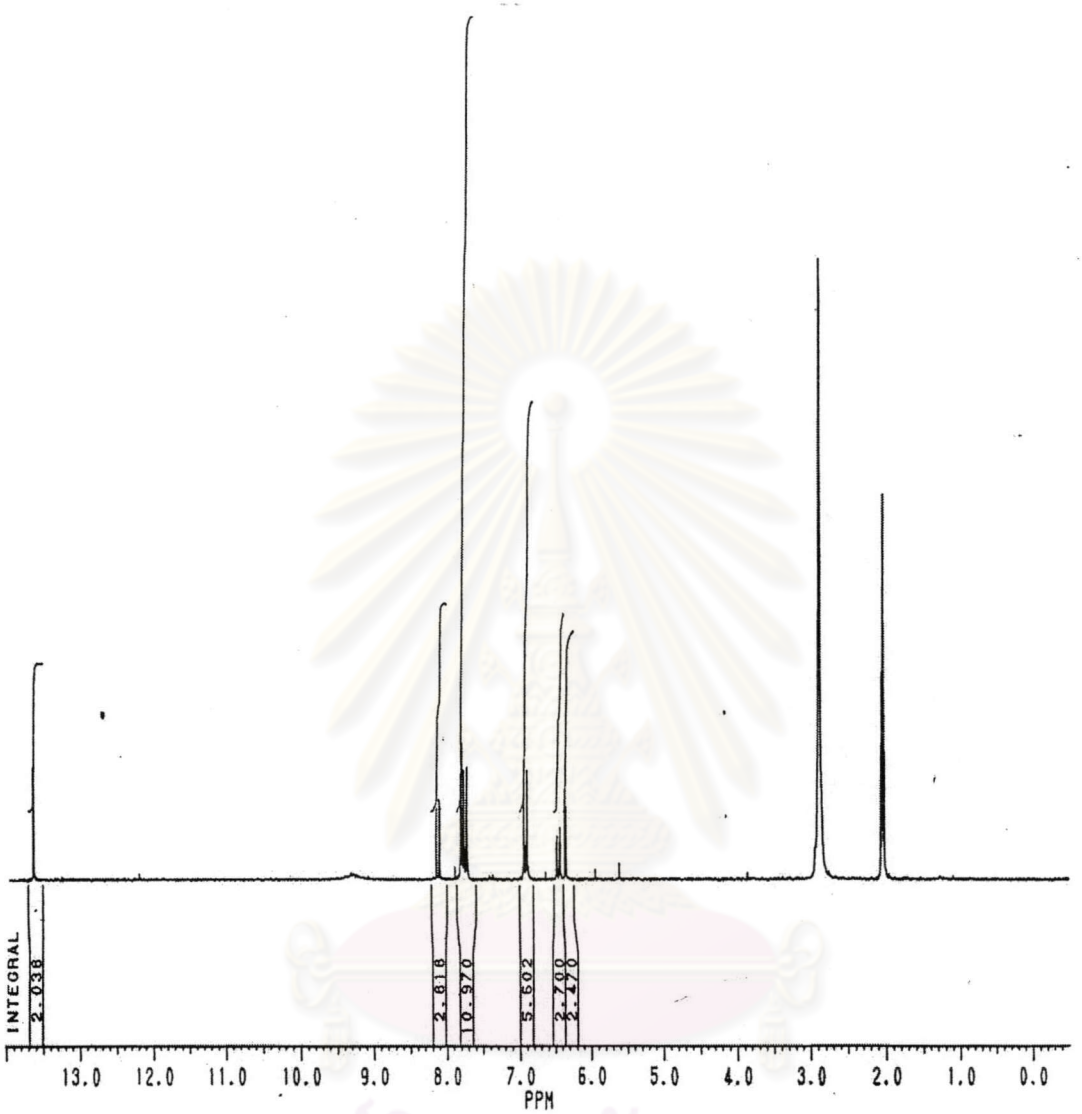


Figure 3.63 ^{1}H-NMR spectrum of compound **11**

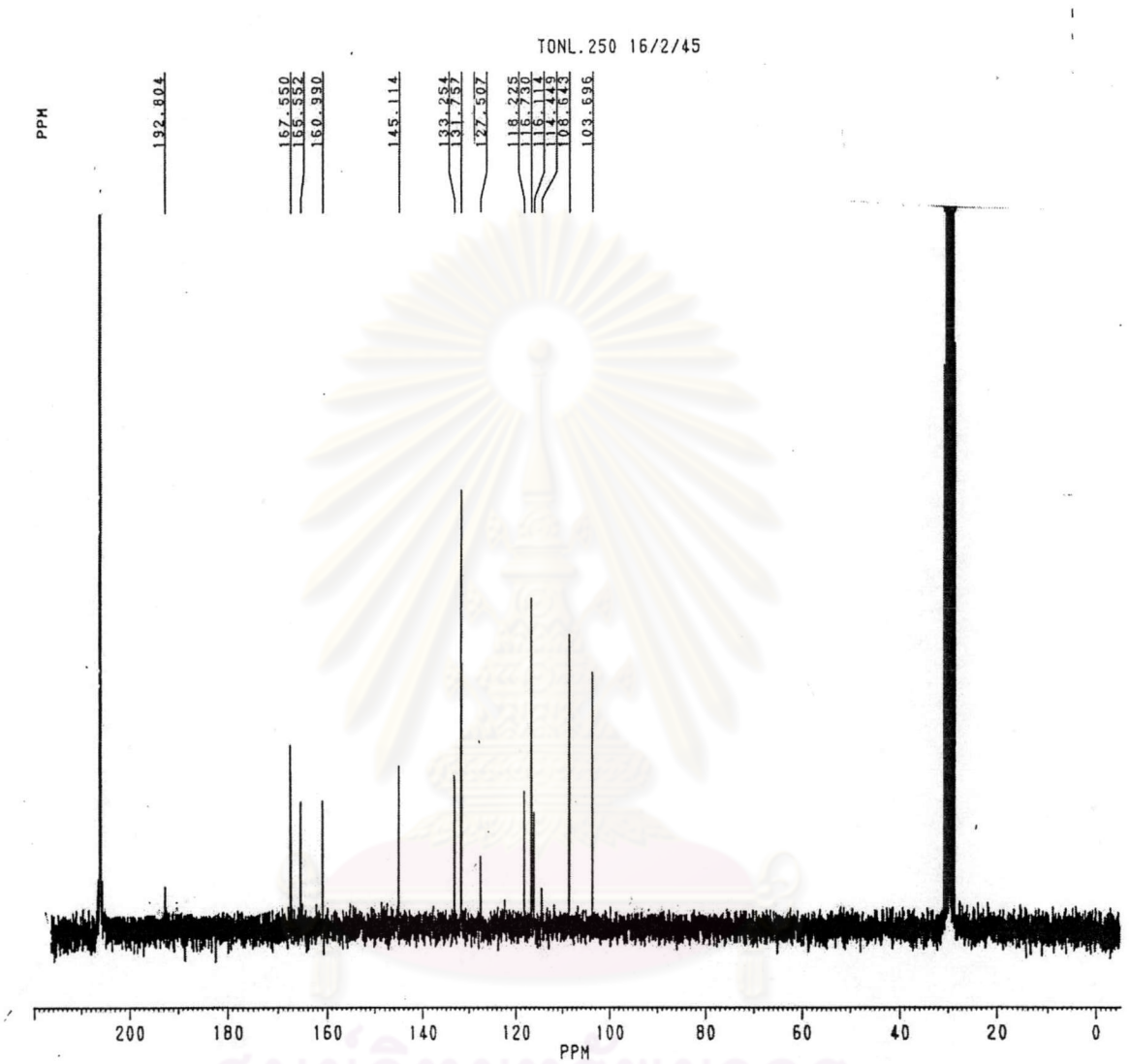


Figure 3.64 ^{13}C-NMR spectrum of compound **11**

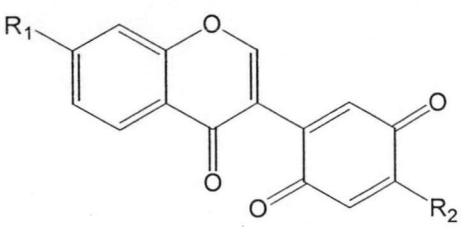
3.2.12 Compound 12

Compound **12** was yielded as yellow needles from open column chromatography of ethyl acetate crude extract. Its melting point was 238-241 ° C and R_f value was 0.24 (SiO_2, EtOAc-CH_2Cl_2, 2:8). Compound **12** was slightly soluble in acetone but not in methanol, ethyl acetate and dichloromethane.

The IR spectrum (**Figure 3.65**) indicated hydroxy group at ν_{max} 3247 cm^{-1} (broad), the strong absorption band at ν_{max} 1629 cm^{-1} revealed the presence of α, β-unsaturated carbonyl. Compound **12** showed molecular ion peak M^+ at m/z 300, providing a molecular formula of $C_{16}H_{12}O_6$, indicating 11 degrees of unsaturation. In the mass spectrum also showed fragment ion peaks at m/z 283, 255, 163 and 108 in the electron impact mass spectrum (EIMS) (**Figure 3.66**).

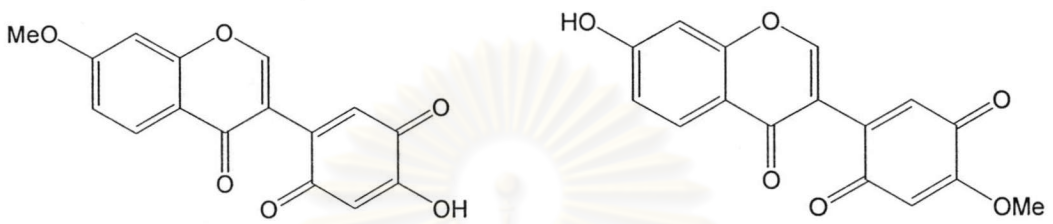
Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum (DMSO-d_6, 500 MHz) (**Figure 3.67**) exhibited ABX-type signals [δ 6.80 (1H, d, J = 2.1 Hz), 6.98 (1H, dd, J = 2.4, 8.85 Hz), and 7.91 (1H, d, J = 8.85 Hz)], two singlet signals at δ 6.24, 7.19 due to aromatic protons and a singlet signal at δ 8.32 due to proton attached to the sp^2 carbon. This means that compound **12** should be an isoflavone or flavone derivative.

In the ^{13}C-NMR spectrum (**Figure 3.68**), fifteen carbon signals were observed due to four oxygenated carbons at δ 158.3 (C-7), 157.1 (C-9), 156.5 (C-2) and 162.9 (C-4'), three quaternary carbons at δ 116.0 (C-10), 116.7 (C-3) and 139.0 (C-1'), five carbons bearing an aromatic proton at δ 132.8 (C-5), 115.6 (C-6), 102.3 (C-8), 108.0 (C-3') and 127.2 (C-6') and three carbonyl carbon at δ 173.4 (C-4), 185.3 (C-2') and 181.5 (C-5') along with one methoxy group at δ 56.5. The two carbonyl carbons (185.3, 181.5) revealed of 1,4-quinone type of ring B. From the evidence of the ^{13}C-NMR spectrum compound **12** was deduced to be an isoflavonequinone derivative which the main structure of this compound was shown below.



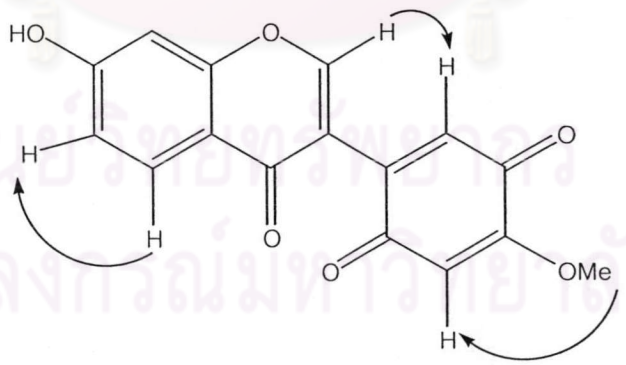
Substituents :
- OH
- OMe

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. The possible structure of this compound can be deduced as follow:



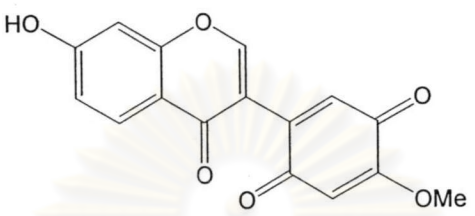
(a) (b)

NOE difference (**Figures 3.69-3.71**) was a useful technique to determine which one was compound 12. Irradiation of the aromatic proton at δ 8.32 resulted NOE enhancement of H-6' (δ 7.05) signal, whereas irradiation of the proton signal at δ 7.91 caused enhancement of the proton signal at δ 6.98 (H-6). However irradiation of the proton signal at δ 3.81 caused enhancement proton signal at δ 6.24 (H-3'). The NOE DIFF correlation of compound **12** was displayed as below.



NOE DIFF correlation of compound **12**

Mainly based upon spectral data, particularly NOE technique, compound **12** was verified as bowdichione. To confirm this structure, the ^{13}C-NMR spectrum of compound **12** was compared with the previously reported data of bowdichione.



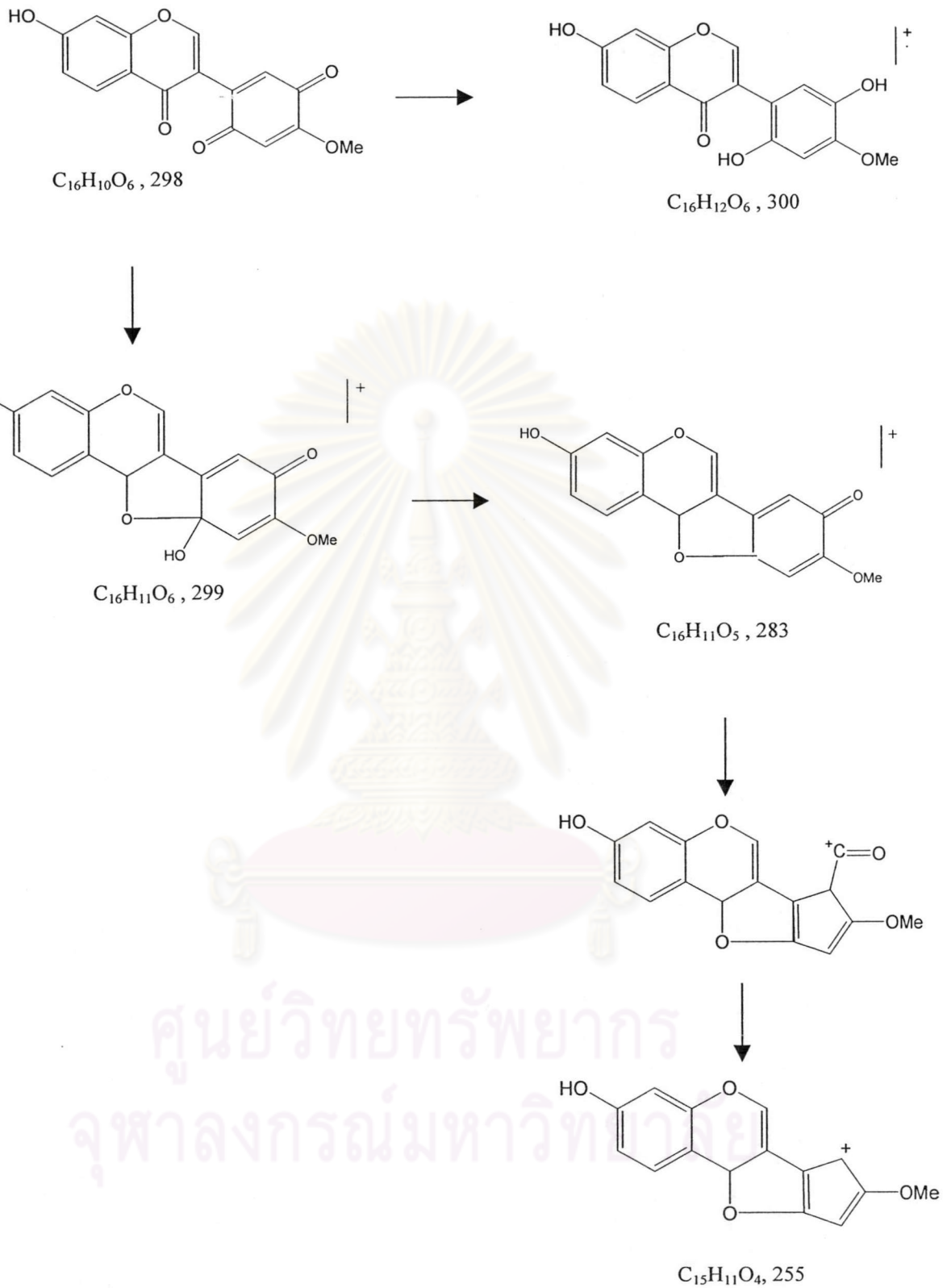
Structure of compound **12** (bowdichione)

Table 3.22 ^{1}H and ^{13}C spectral data of compound **12**

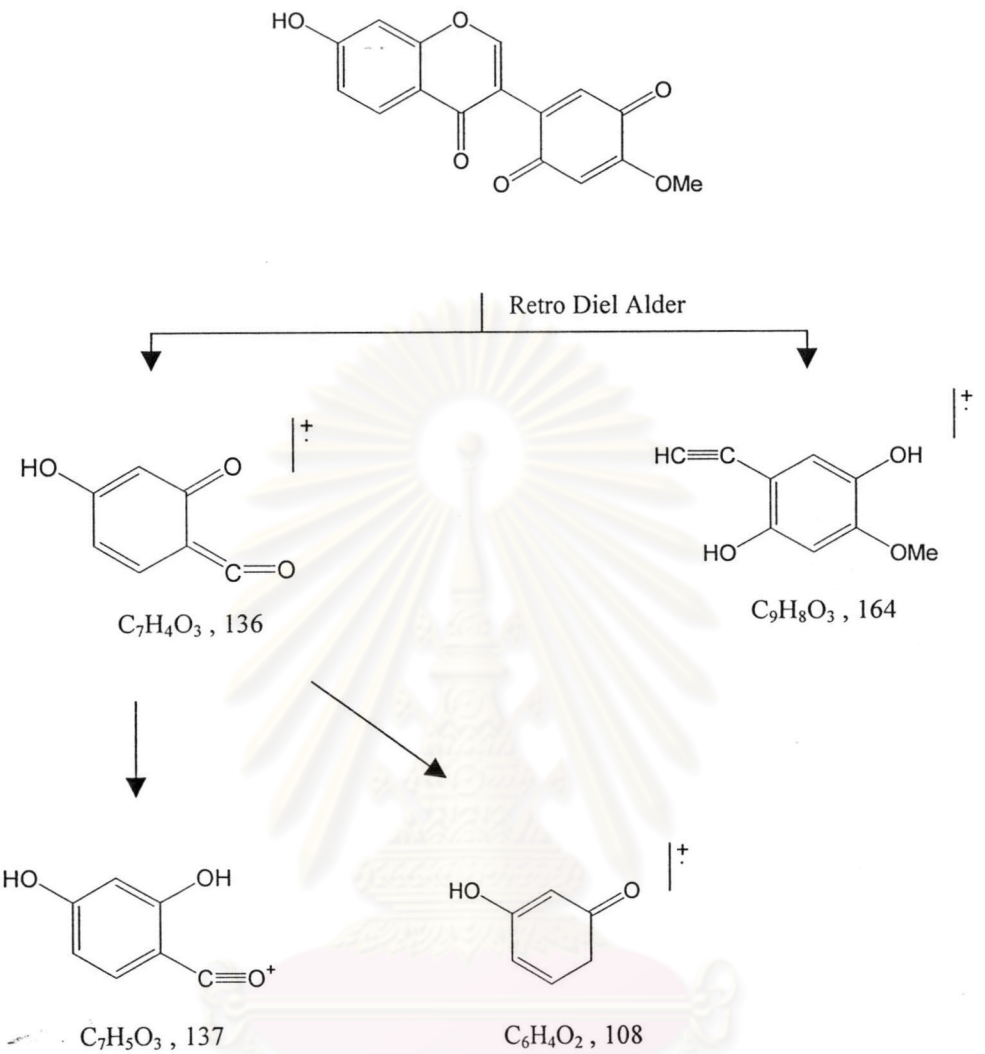
position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	156.5	8.31	1H, s
3	116.7		
4	173.4		
5	132.8	7.91	1H, d, J = 8.85 Hz
6	115.6	6.98	1H, dd, J = 2.45, 8.85 Hz
7	158.3		
8	102.3	6.80	1H, d, J = 2.14 Hz
9	157.1		
10	116.0		
1'	139.0		
2'	185.3		
3'	108.0	6.24	1H, s
4'	162.9		
5'	181.5		
6'	127.2	7.05	1H, s
OMe	56.5	3.81	3H, s

Table 3.23 ^{13}C NMR data of compound **12** compared with Bowdichione.[22]

Position	Compound **12**	Bowdichione
2	156.5	156.7
3	116.7	116.9
4	173.4	173.7
5	132.8	133.0
6	115.6	115.8
7	158.3	158.6
8	102.3	102.5
9	157.1	157.4
10	116.0	116.2
1'	139.0	139.3
2'	185.3	185.5
3'	108.0	108.1
4'	162.9	163.2
5'	181.5	181.8
6'	127.2	127.4
OMe	56.5	56.6



Scheme 3.12 Possible mass fragmentation patterns of compound **12**



Scheme 3.12 Possible mass fragmentation patterns of compound **12** (continued)

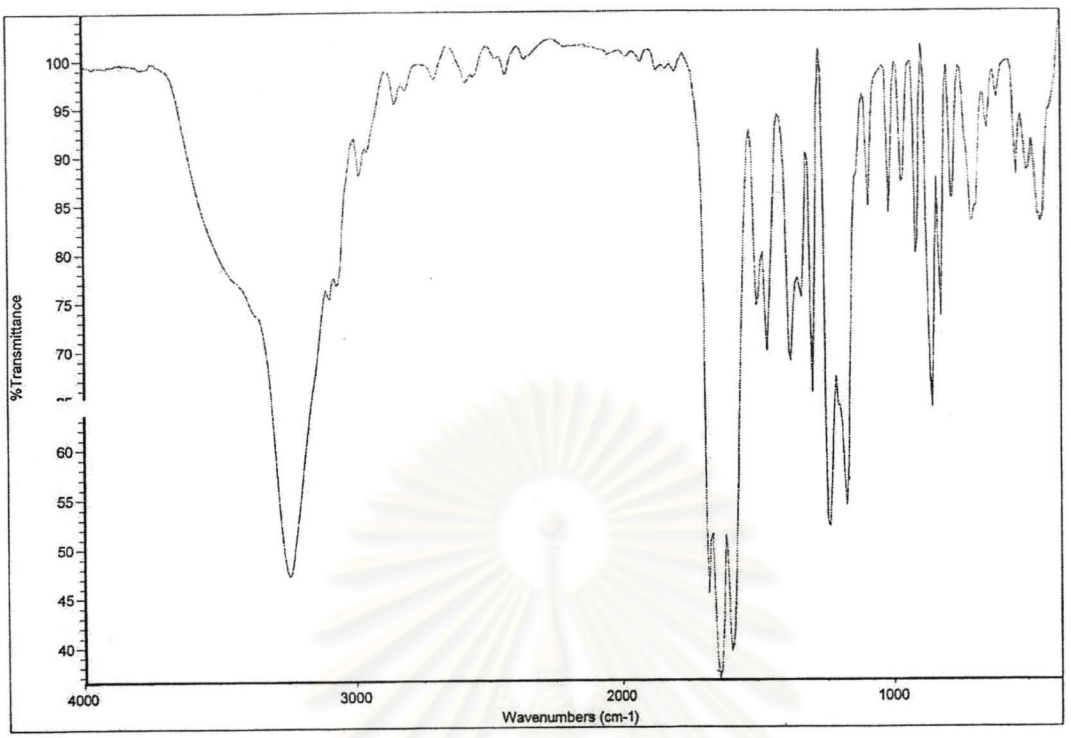


Figure 3.65 IR spectrum of compound **12**

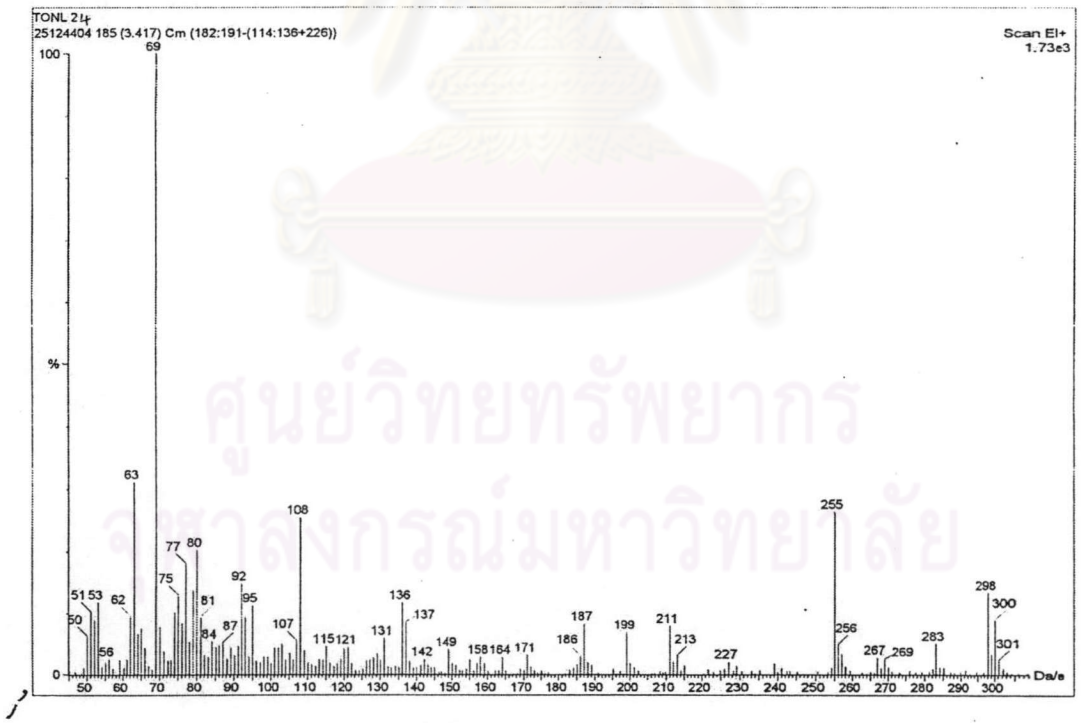


Figure 3.66 Mass spectrum of compound **12**

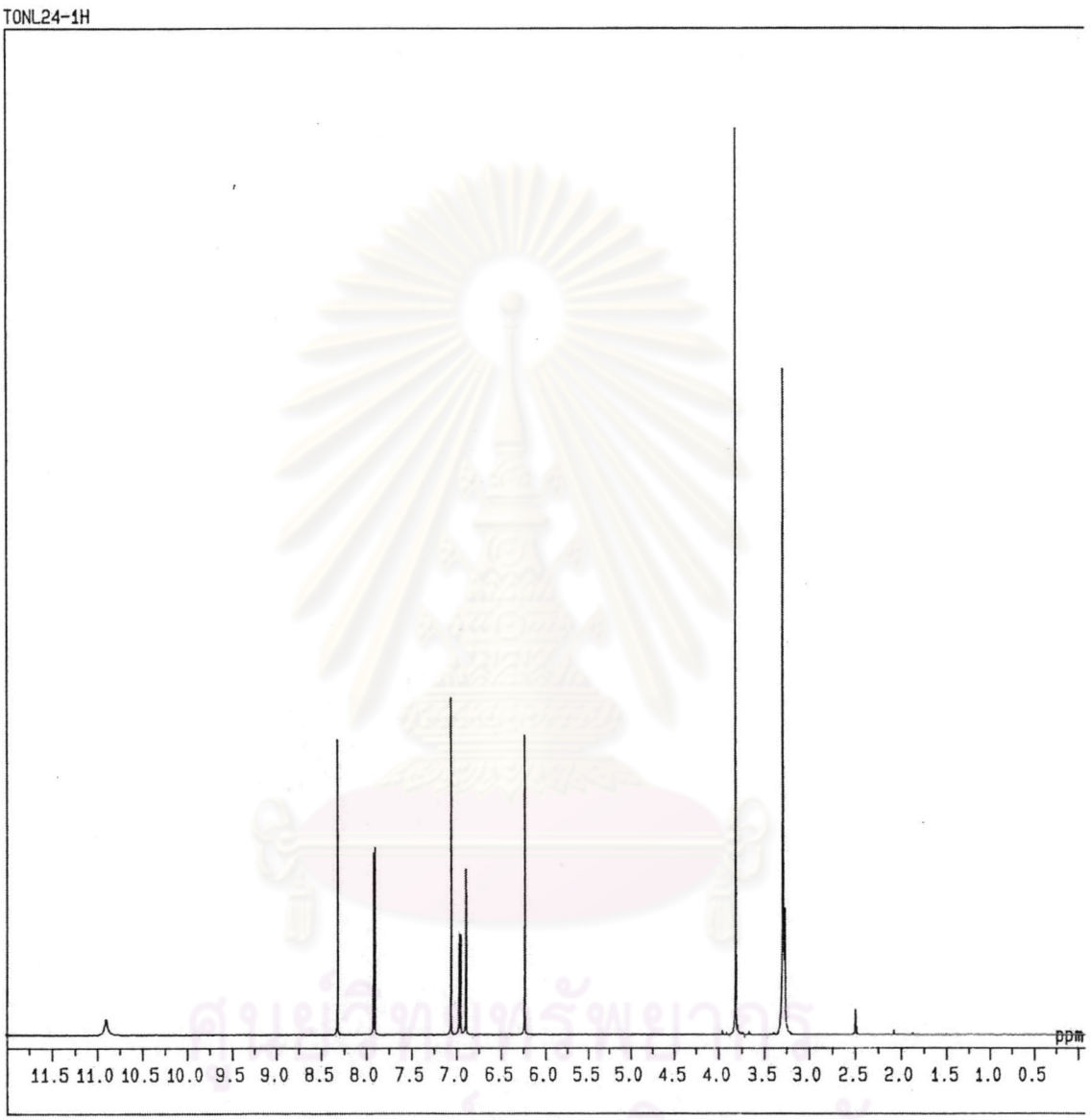


Figure 3.67 ^{1}H-NMR spectrum of compound **12**

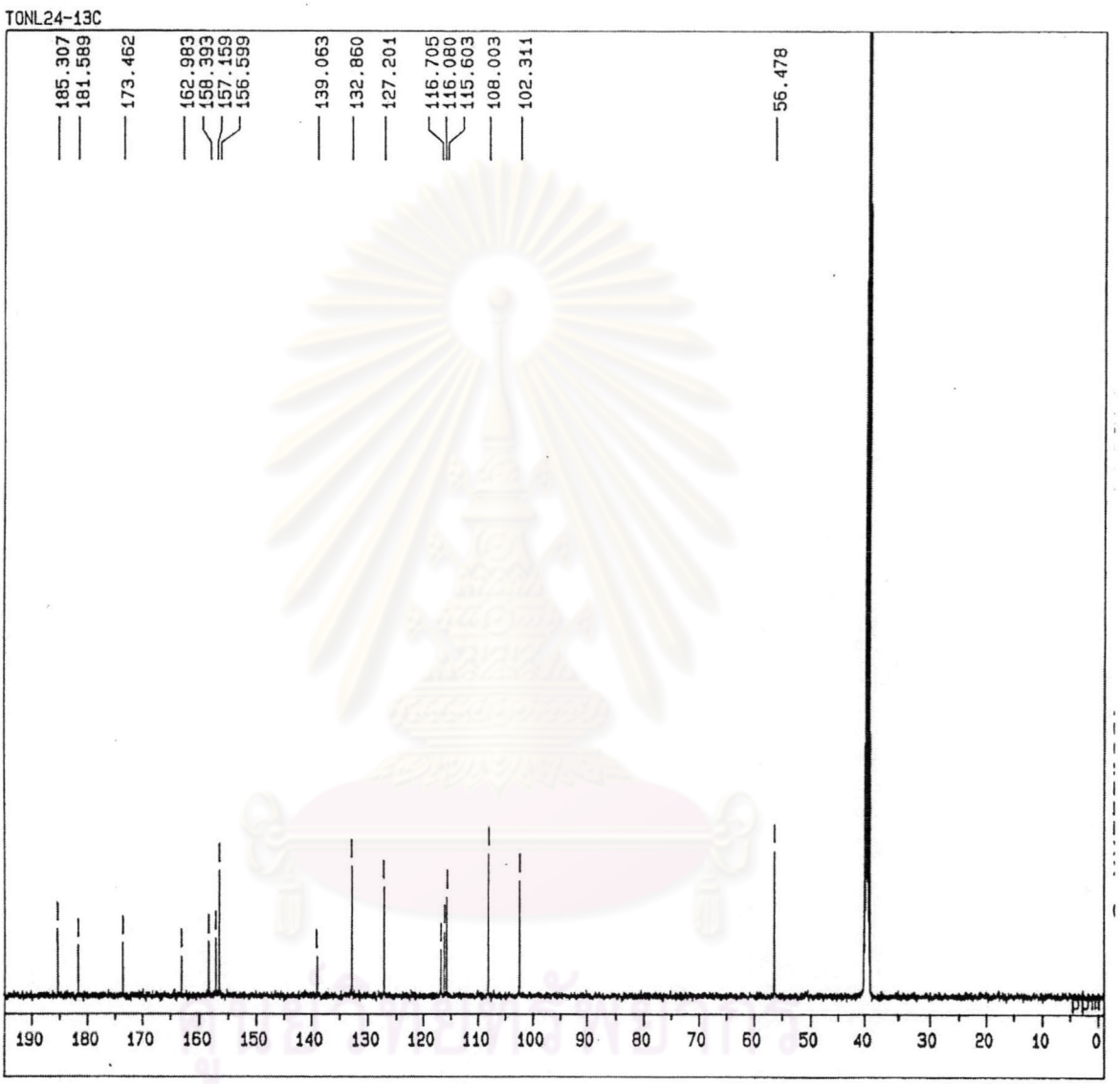


Figure 6.68 ^{13}C-NMR spectrum of compound **12**

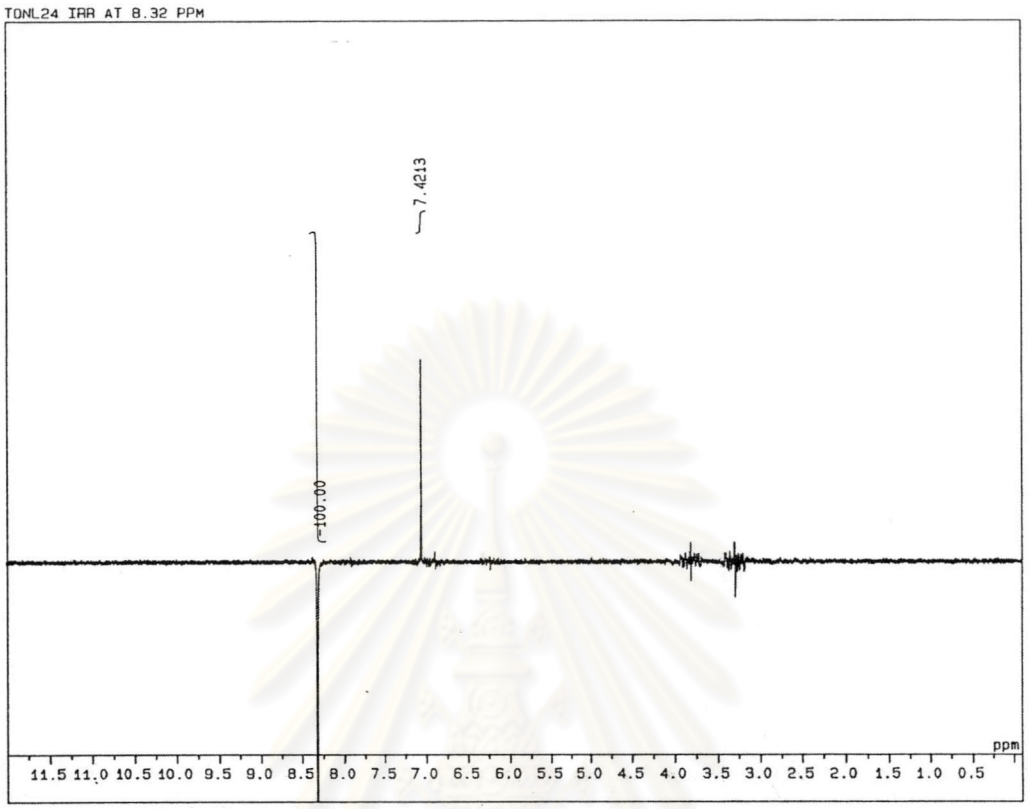


Figure 6.69 NOE DIFF of compound **12** (irradiated at 8.31 ppm)

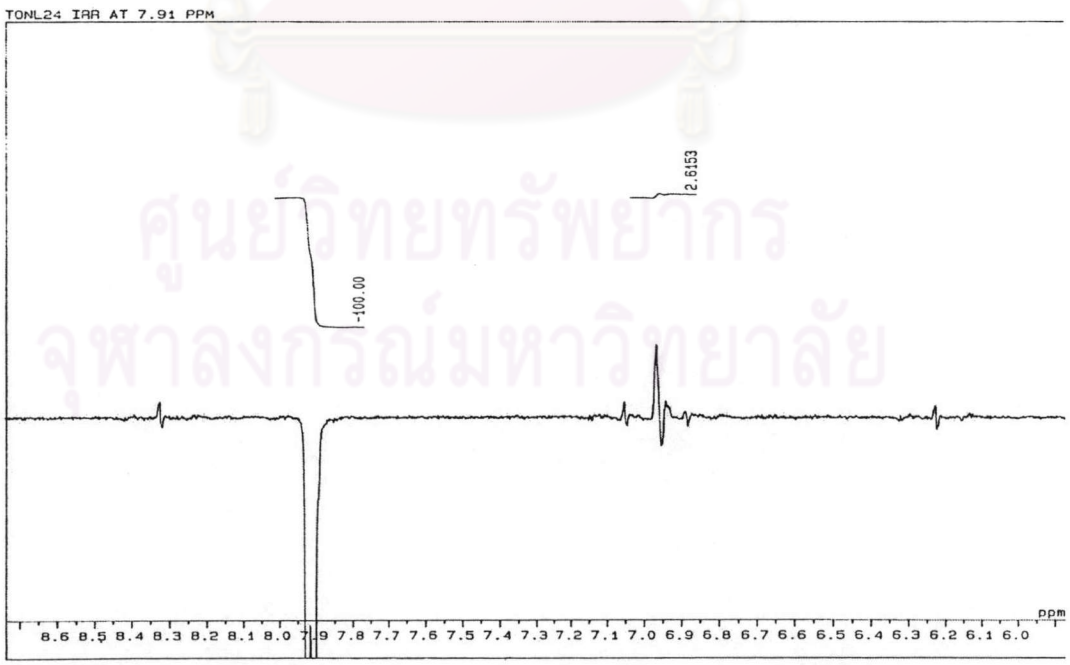


Figure 6.70 NOE DIFF of compound **12** (irradiated at 7.91 ppm)

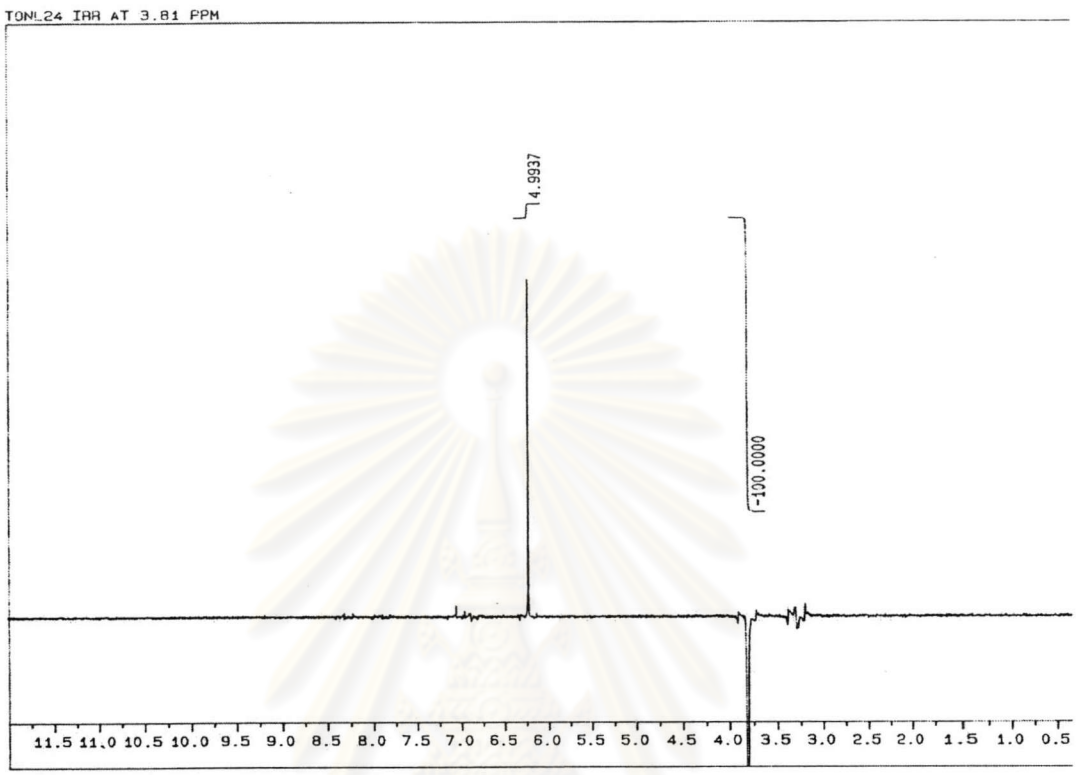


Figure 6.71 NOE DIFF of compound **12** (irradiated at 3.81 ppm)

3.2.13 Compound 13

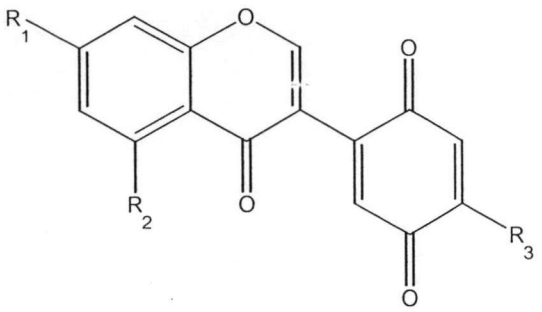
Compound **13** appeared as light yellow needles, decomposed at 250 °C, from open column chromatography of ethyl acetate crude extract and further purified by recrystallization in acetone. R_f value was 0.58 (SiO_2, EtOAc-CH_2Cl_2, 6:4). Compound **13** was slightly soluble in acetone and methanol but not in ethyl acetate and dichloromethane.

The IR spectrum (**Figure 3.73**) indicated the presence of hydroxyl group at ν_{max} 3370 cm^{-1}, carbonyl group which conjugated with double bond and hydrogen-bonded with hydroxyl group at ν_{max} 1649 cm^{-1} and benzene rings at ν_{max} 1582, 1450 and 1321 cm^{-1}.

The mass spectrum (**Figure 3.74**) displayed a molecular ion [M^+] peak at m/z 314 along with fragment ions at m/z 299, 271, 153, 164 and 124.

The ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz) (**Figure 3.75**) clearly showed signals for five aromatic protons. Two one-proton doublets meta coupling to each other at δ 6.45 and 6.31 (d, J = 2.1 Hz) were assigned to H-6 and H-8 of ring A, two singlet signals at δ 6.15 and 7.14 due to aromatic proton at ring B which assigned to H-6' and H-3' and one proton attached to sp^2 carbon at δ 8.30. This mean that compound **13** should be characteristic of an isoflavone or flavone derivative.

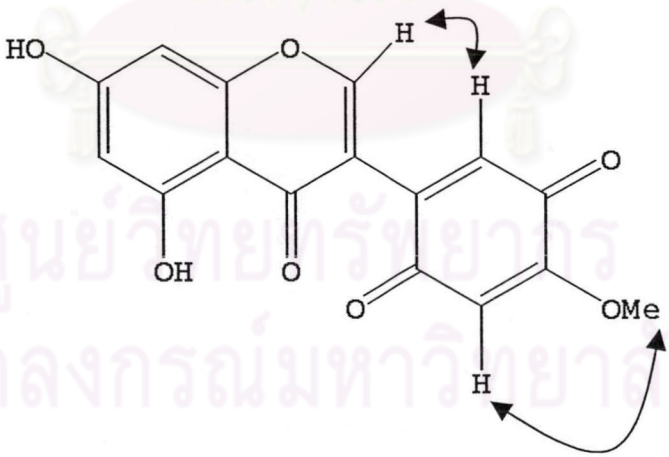
The ^{13}C-NMR spectrum (**Figure 3.76**) of this compound appeared sixteen carbons of fifteen skeletons and one methoxy group (characteristic of flavonoid). This compound contained seven quaternary carbons at δ 161.1 (C-5), 164.7 (C-7), 158.4 (C-9), 103.9 (C-10), 115.4 (C-3), 137.9 (C-1') and 157.1 C-4'), five methine carbons at δ 99.4 (C-6), 94.1 (C-8), 157.4 (C-2), 115.4 (C-3') and 133.2 (C-6') and three carbonyl carbons at δ178.7 (C-4), 184.9 (C-2') and 181.4 (C-5') together with one methoxy group at δ 56.5. From this evidence, the two carbonyl carbon (184.9, 181.4) indicated of 1, 4-quinone type of ring B. The molecular formula of compound **13** was determined as $C_{16}H_{10}O_7$ from its EIMS as its NMR spectra, corresponding to 12 degrees of unsaturation. All of the above data suggested that the possibility of a flavonoid skeleton of isoflavonequinone type. On the basis of NMR spectra and comparison with the reported isoflavone quinone indicated that the molecule of this compound had the basic skeleton as shown below:



Substituents :

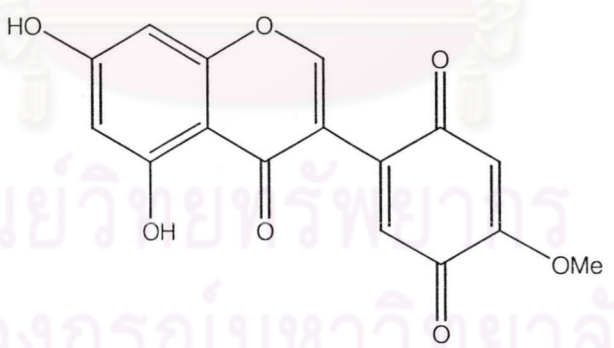
- OH (2)
- OMe (1)

The NOE DIFF experiments were used to obtain additional information. Irradiation of the proton signal at δ 7.14 (H-6') only caused enhancement of the proton signal at δ 8.30 (H-2); irradiation of the proton signal at δ 6.15 (H-3') resulted in NOE enhancement of methoxy signal and irradiation of the proton signal at δ 3.90 caused enhancement of the proton signal at δ 6.15 (H-3') (**Figures 3.77-3.79**). The NOE DIFF correlation of compound **13** was displayed below.



NOE DIFF correlation of compound **13**

In addition, the structure of compound **13** was also confirmed by single crystal X-ray diffraction analysis. Single crystal of this compound was prepared by recrystallization in acetone. After 1 week, light yellow rod-shaped crystals had grown by slow solvent evaporation. A crystal, 0.4 x 0.5 x 0.7 mm^3 in dimensions, was mounted in quartz capillary and used for X-ray data collection performed at room temperature with a Bruker AXs SMART diffractometer equipped with a CCD area detector using graphite-monochromated MoKα radiation (λ = 0.71073 A°). The data were collected in the θ-range 1.50 – 30.50 °. The crystal belongs to the monoclinic space group $P2_1/a$ with unit cell dimensions a = 12.6447 (2), b = 7.70440 (10), c = 13.55730 (10) A° and β = 92.1032 (9) °. The formula per asymmetric unit is $C_{16}H_{10}O_7$, (**Table 3.25**). The bond distances and bond angle were also shown in **Table 3.26** and **3.27**. A completion of all X-ray data led to the solution of the structure of compound **13** (**Figure 3.72**). Hydrogen bonding between the phenolic group at C-5 (ring A) and oxygen of the C-4 ketonic carbonyl were also apparent in the X-ray structure. Based on these spectroscopic observation and single crystal X-ray diffraction analysis, the structure of compound **13** was found to be 5,7-dihydroxy-4'-methoxyisoflavonequinone. From our knowledge, this is a new compound.



The structure of compound **13**

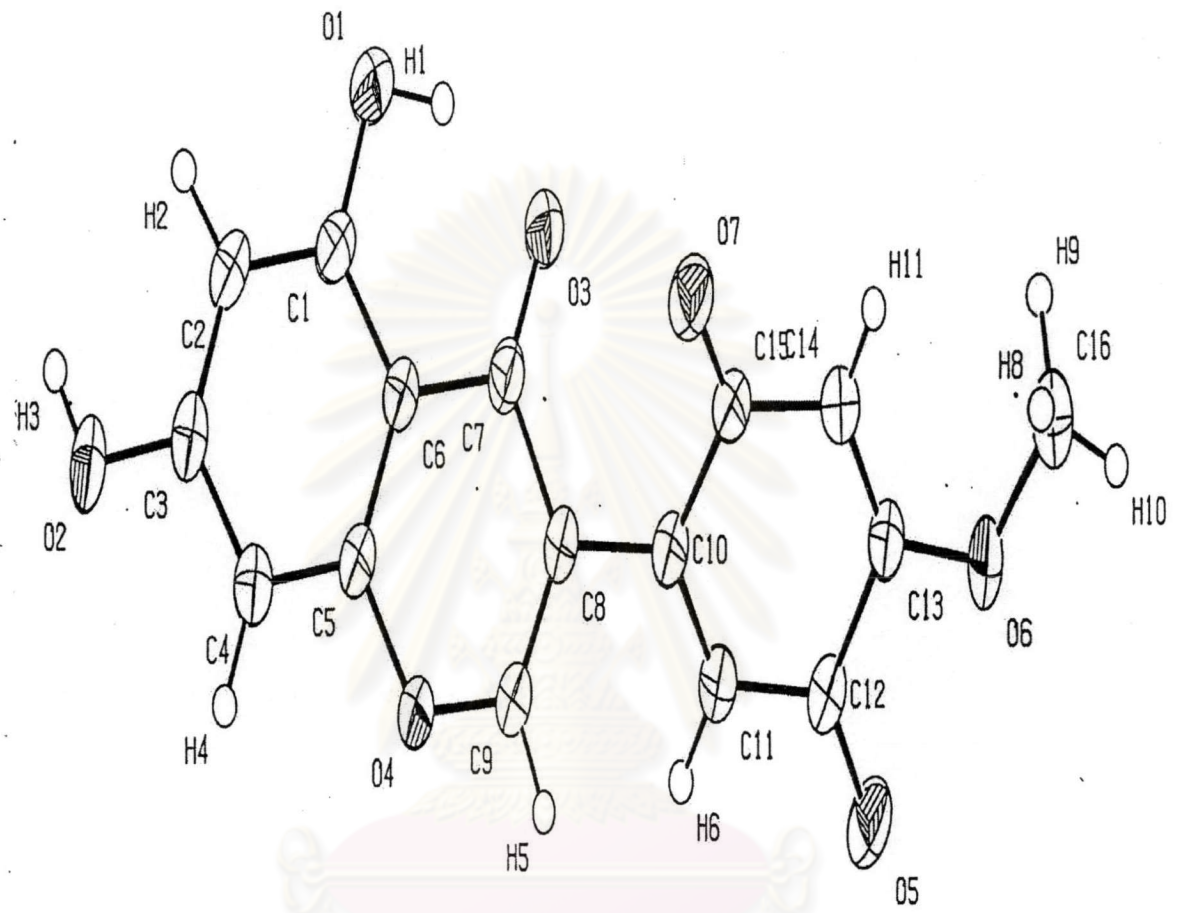


Figure 3.72 Computer-generated ORTEP drawing of compound **13**

Table 3.24 ^{1}H and ^{13}C NMR spectral data of compound **13**

position	δ (ppm)		No. of proton, multiplicity and coupling constants
	^{13}C	^{1}H	
2	157.4	8.30	1H, s
3	115.4		
4	178.7		
5	161.1		
6	99.4	6.45	1H, d, J = 2.13 Hz
7	164.7		
8	94.1	6.31	1H, d, J = 2.13 Hz
9	158.4		
10	103.9		
1'	137.9		
2'	184.9		
3'	107.9	6.15	1H, s
4'	157.1		
5'	181.4		
6'	133.2	7.14	1H, s
OMe	56.5	3.90	3H, s

Table 3.25 Crystallographic Data; Data Collection, Solution and Refinement for compound **13**

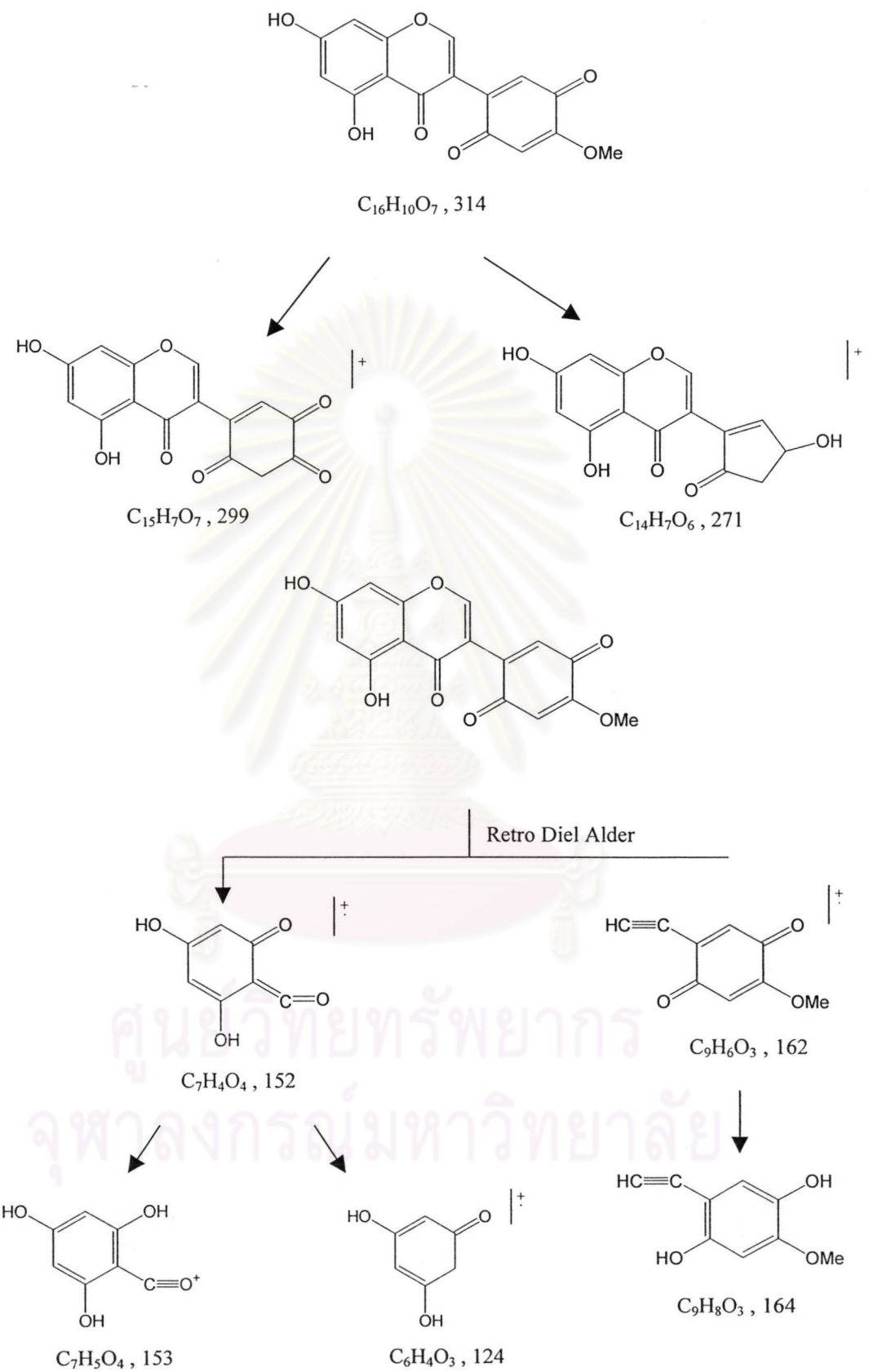
Chemical formula	$C_{16}H_{10}O_7$
Formula weight	314.24
Crystal habit, color	Rod, light yellow
Cyystal size (mm^3)	0.4 x 0.5 x 0.7
Crystal systal	Monoclinic
Space group	$P2_1/a$
Unit cell dimensions	
a (A°)	12.6447 (2)
b (A°)	7.70440 (10)
c (A°)	13.55730 (10)
β (deg)	92.1032 (9)
Volume $(A°)^3$	1319.86 (3)
Z	4
D_x ($g\ cm^{-1}$)	1.581
μ (mm^{-1})	0.127
F (000)	648
Diffractometer	Bruker, CCD
Wavelength (A°)	MoKα; 0.71073
Temperature (°C)	Room temperature
Θ rang for data collection (deg)	1.50 to 30.50
Resolution (A°)	
Index range	$-17 < h < 15, -10 < k < 10, -16 < l < 18$
Structure solution	ab initio method (SHELXD)
Refinement method	Full-metrix least-aquares on F^2
Data / parameters	3774 / 248
R (F^2)	0.0430
Goodness of fit	1.020

Table 3.26 Bond angles of compound **13** from X-ray diffraction analysis.

	Angle (deg)		Angle (deg)
C6 - C1 - O1	120.13	C8 - C10 - C11	122.42
C2 - C1 - O1	119.33	C11 - C10 - C15	119.60
C2 - C1 - C6	120.53	C10 - C11 - H6	122.11
C1 - C2 - H2	119.09	C10 - C11 - C12	122.07
C1 - C2 - C3	119.59	C12 - C11 - H6	115.71
C3 - C2 - H2	121.32	C11 - C12 - O5	121.87
C2 - C3 - O2	122.00	C11 - C12 - C13	117.30
C2 - C3 - C4	121.77	C13 - C12 - O5	120.83
C4 - C3 - O2	116.22	C12 - C13 - O6	111.89
C3 - C4 - H4	121.89	C12 - C13 - C14	121.09
C3 - C4 - C5	117.46	C14 - C13 - O6	127.00
C5 - C4 - H4	120.55	C13 - C14 - H11	124.45
C4 - C5 - O4	116.32	C13 - C14 - C15	121.08
C4 - C5 - C6	123.39	C15 - C14 - H11	114.33
C6 - C5 - O4	120.29	C10 - C15 - C14	118.39
C1 - C6 - C5	117.14	C14 - C15 - O7	120.73
C5 - C6 - C7	121.37	C10 - C15 - O7	120.81
C1 - C6 - C7	121.42	H9 - C16 - H10	114.23
C6 - C7 - O3	121.84	H8 - C16 - H10	107.80
C6 - C7 - C8	114.73	H8 - C16 - H9	110.61
C8 - C7 - O3	123.35	O6 - C16 - H10	101.93
C7 - C8 - C10	121.09	O6 - C16 - H9	110.57
C7 - C8 - C9	119.48	O6 - C16 - H8	111.42
C9 - C8 - C10	119.35	C1 - O1 - H1	103.39
C8 - C9 - H5	124.30	C3 - O2 - H3	111.55
C8 - C9 - O4	125.19	C5 - O4 - C9	118.88
O4 - C9 - H5	110.50	C13 - O6 - C16	115.72
C8 - C10 - C15	117.89		

Table 3.27 Bond distances of compound **13** from X-ray diffraction analysis

	Distance (A°)		Distance (A°)
C1 - C2	1.3833(17)	C10 - C11	1.3438(17)
C1 - C6	1.4194(17)	C10 - C15	1.5020(16)
C1 - O1	1.3444(16)	C11 - C12	1.4725(17)
C2 - C3	1.3902(21)	C11 - H6	0.9709(169)
C2 - H2	0.9621(196)	C12 - C13	1.4982(18)
C3 - C4	1.3901(20)	C12 - O5	1.2218(17)
C3 - O2	1.3552(17)	C13 - C14	1.3358(18)
C4 - C5	1.3811(18)	C13 - O6	1.3459(15)
C4 - H4	0.9869(204)	C14 - C15	1.4685(17)
C5 - C6	1.3954(17)	C14 - H11	0.9292(172)
C5 - O4	1.3761(15)	C15 - O7	1.2220(16)
C6 - C7	1.4521(15)	C16 - O6	1.4503(21)
C7 - C8	1.4607(16)	C16 - H8	1.0256(230)
C7 - O3	1.2449(15)	C16 - H9	1.0000(232)
C8 - C9	1.3426(17)	C16 - H10	0.9162(264)
C8 - C10	1.4817(15)	O1 - H1	0.9641(260)
C9 - O4	1.3518(15)	O2 - H3	0.8298(277)
C9 - H5	0.9286(170)		



Scheme 3.13 Possible mass fragmentation patterns of compound **13**

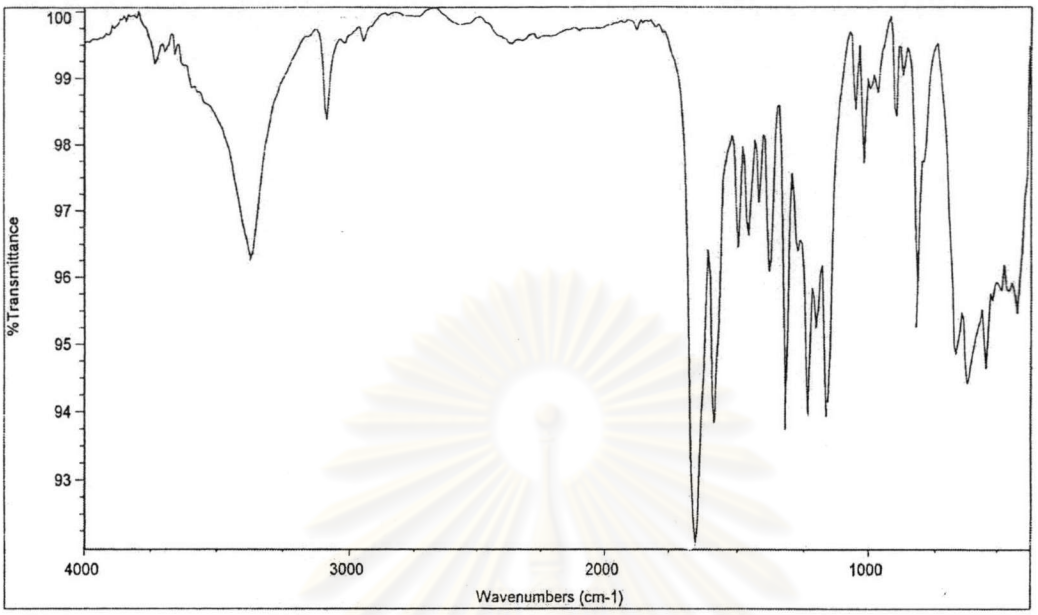


Figure 3.73 IR spectrum of compound **13**

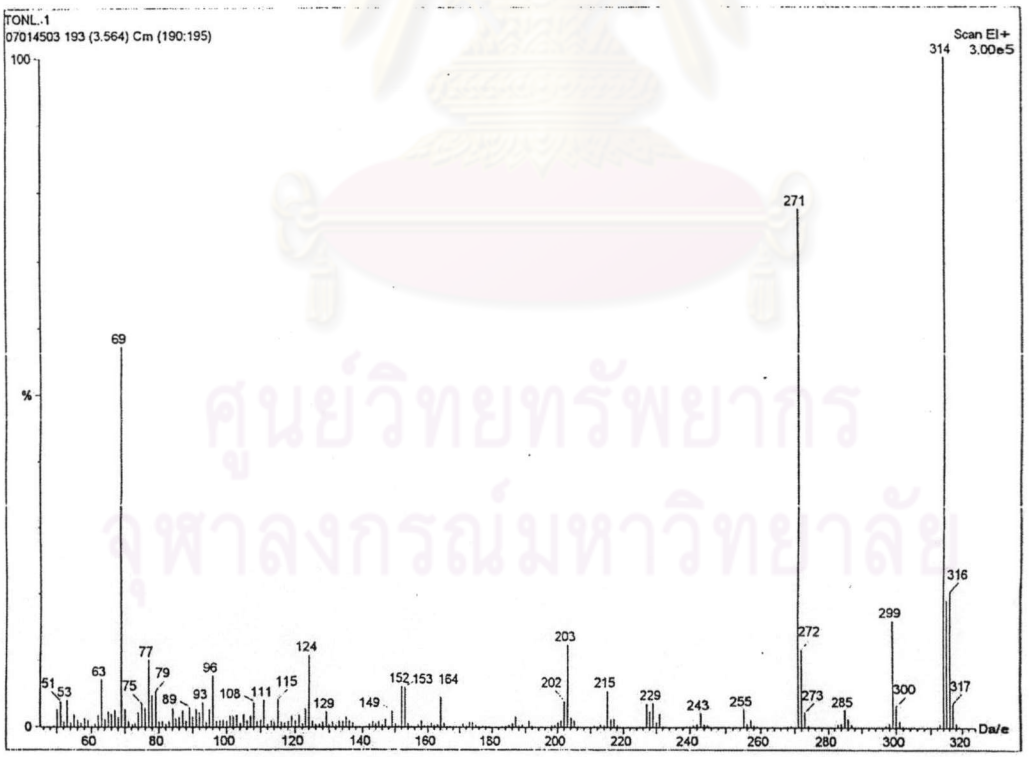


Figure 3.74 Mass spectrum of compound **13**

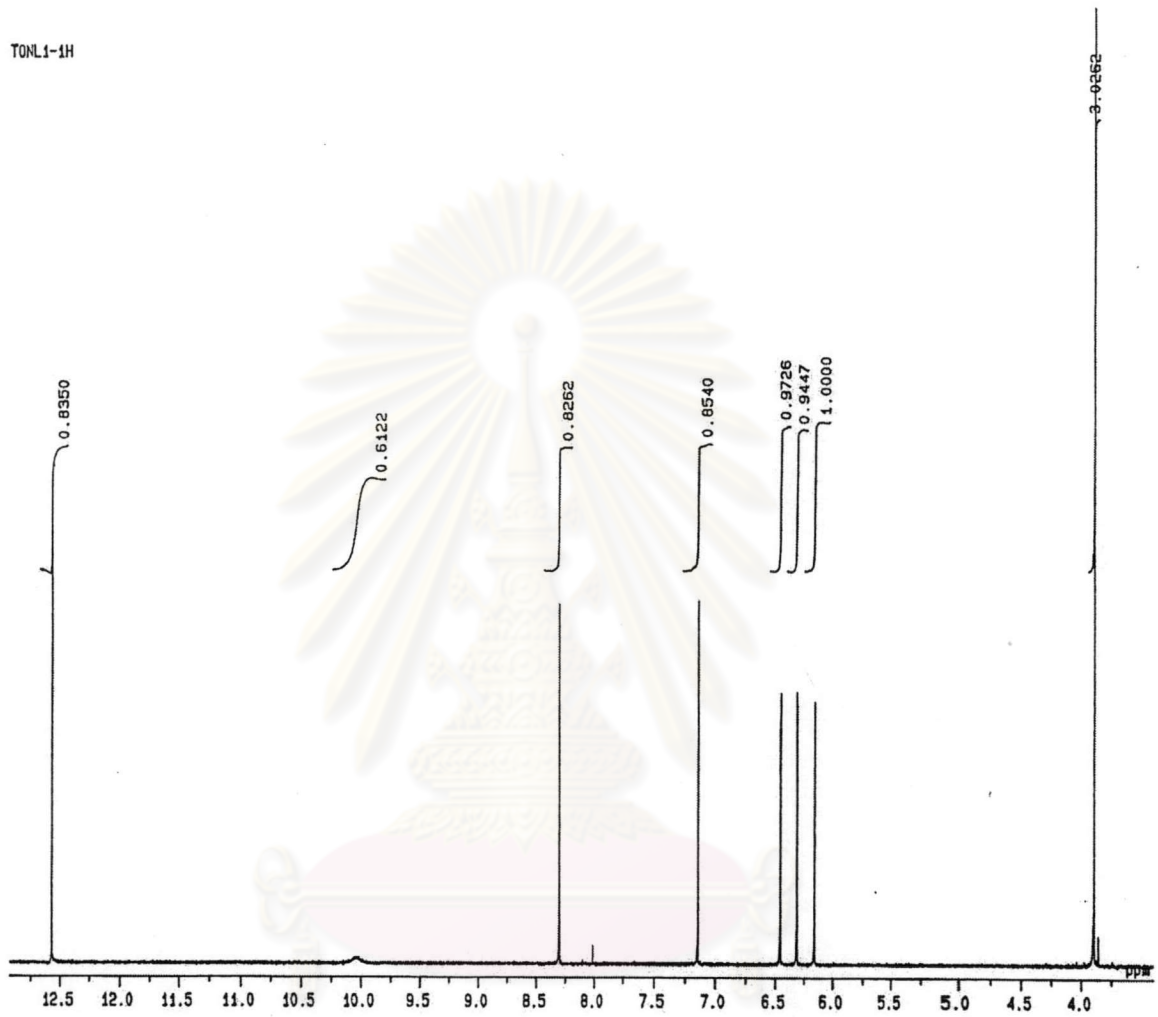


Figure 3.75 ^{1}H-NMR spectrum of compound **13**

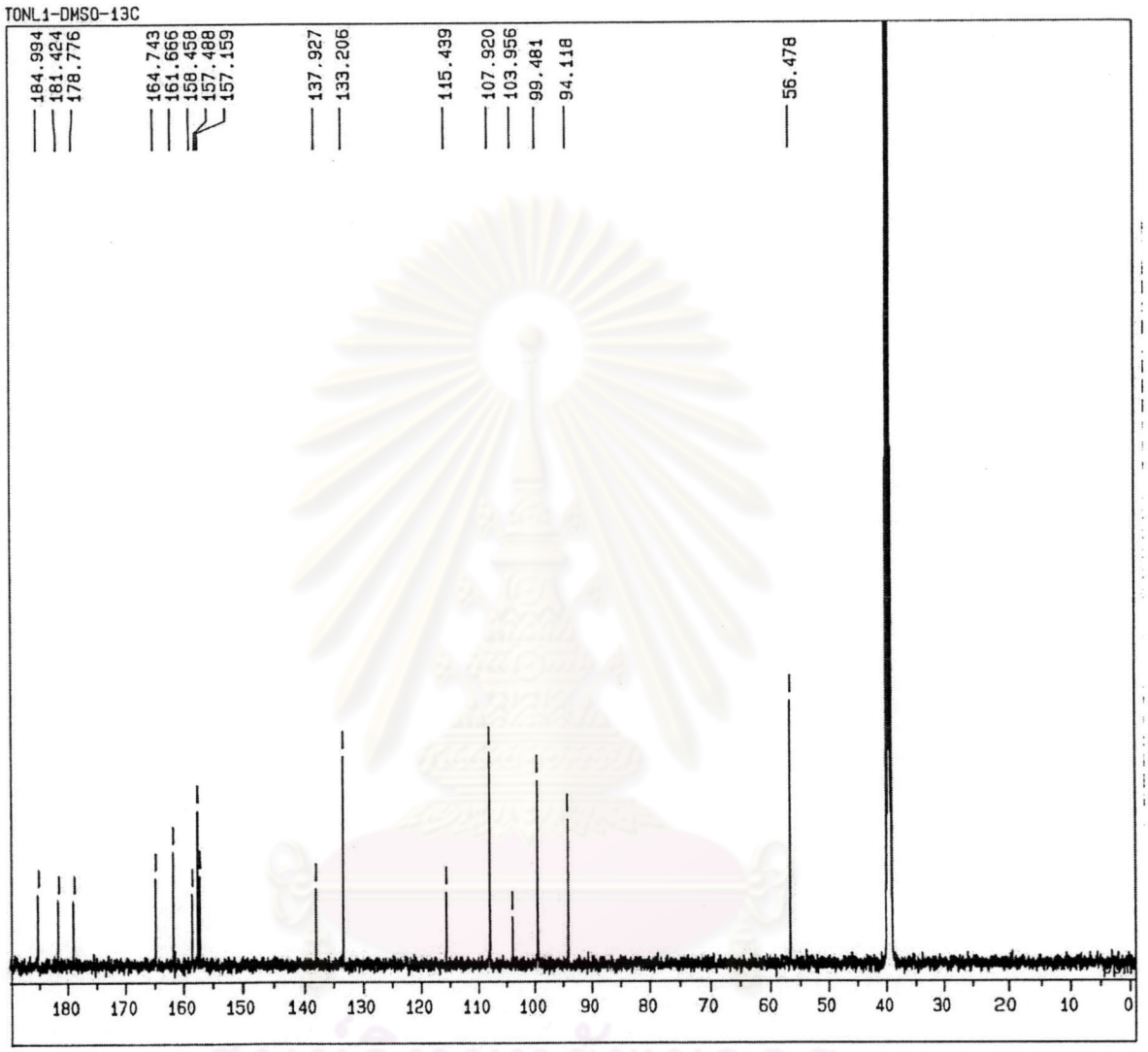


Figure 6.76 ^{13}C-NMR spectrum of compound **13**

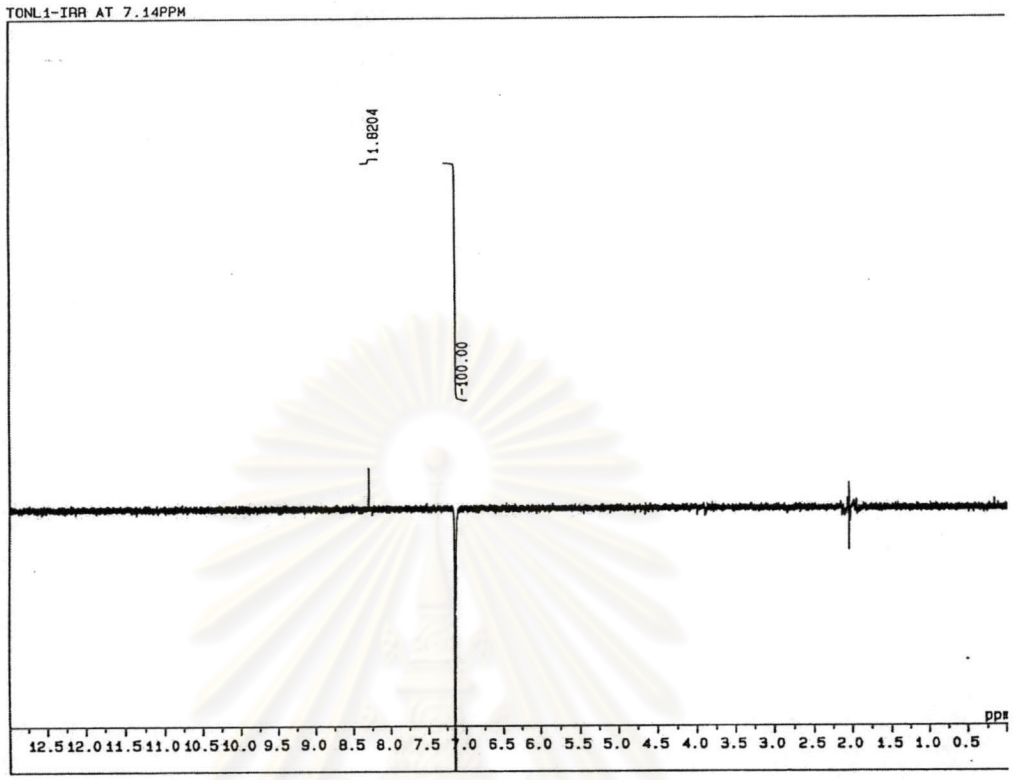


Figure 6.77 NOE DIFF of compound **13** (irradiated at 7.14 ppm)

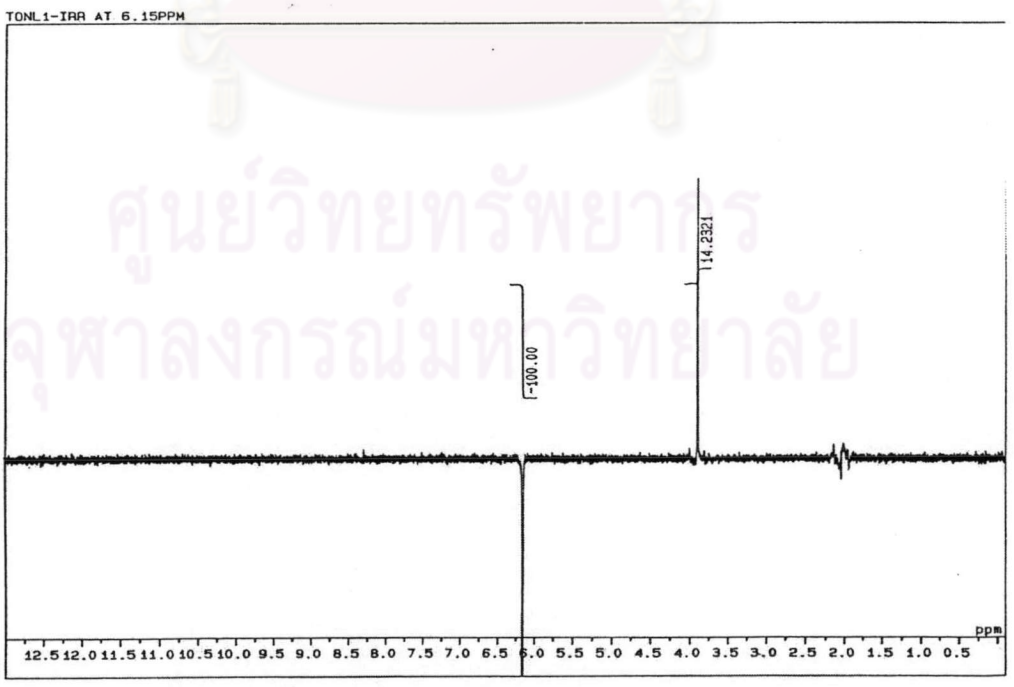


Figure 6.78 NOE DIFF of compound **13** (irradiated at 6.15 ppm)

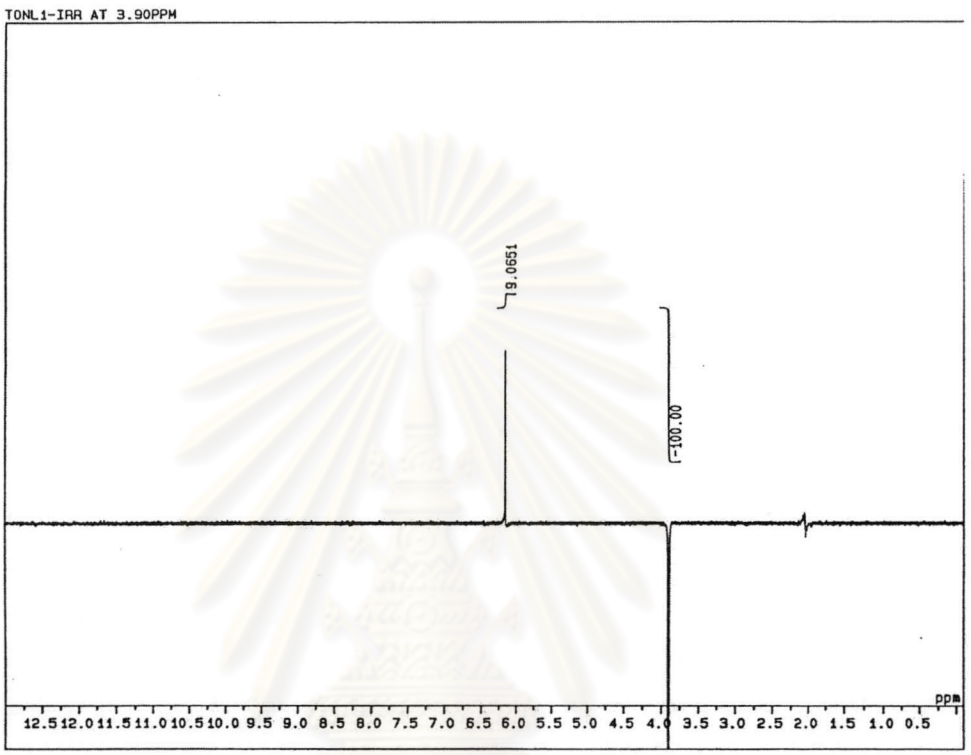


Figure 6.79 NOE DIFF of compound **13** (irradiated at 3.90 ppm)

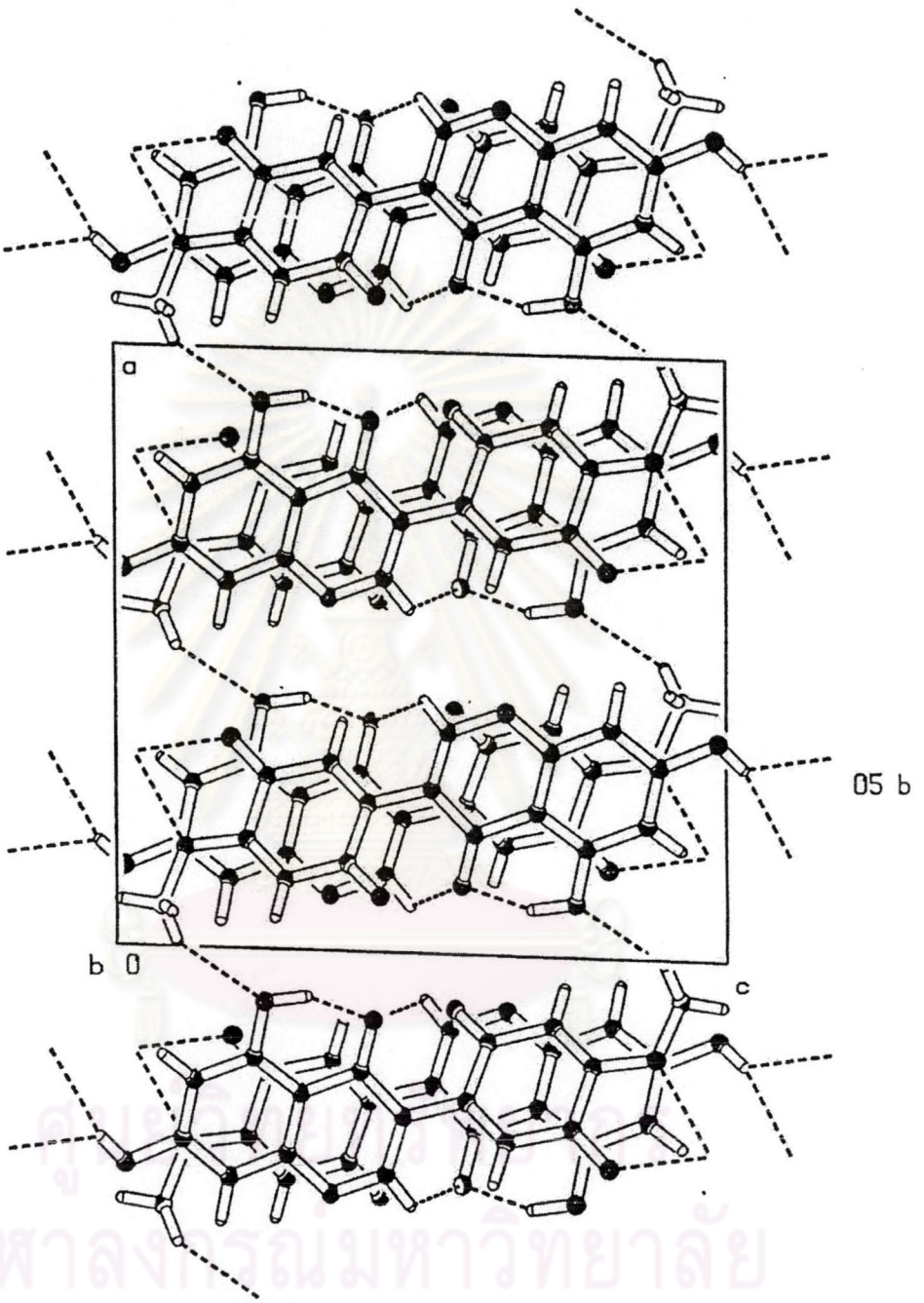


Figure 6.80 Plot of crystal packing of compound **13**

3.3 The biological activity of isolated compounds from *D. cochinchinensis*

The in-vitro IC_{50} values of KB cell lines, free radical scavenging activity and $O_2^{-\cdot}$ scavenging activity of isolated compounds from *D. cochinchinensis* are shown in **Table 3.28, 3.29** and **3.31**, respectively.

Table 3.28 Cytotoxic activity ggainst KB cell lines by MTT assay*

Compound	IC_{50} (μg / ml) λ_{550} nm
1	37
2	65
3	28
4	55
5	9
6	47
7	40
8	25
9	40
10	14
11	8
12	29
13	54

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

Table 3.29 Free radical scavenging activity of isolated compounds on DPPH (0.25 mM)

Sample	Conc. (mM)	% radical scavenging
Compound **1**	1.000	8.71
	0.500	4.37
	0.250	1.95
	0.125	0.53
Compound **2**	1.000	61.71
	0.500	48.25
	0.250	35.34
	0.125	24.02
	0.062	15.04
Compound **3**	1.000	57.85
	0.500	46.30
	0.250	32.37
	0.125	20.00
	0.062	11.76
Compound **4**	1.000	22.08
	0.500	15.82
	0.250	13.64
	0.125	9.60
Compound **5**	1.000	9.85
	0.500	6.23
	0.250	2.42
	0.125	0.70
Compound **6**	1.000	67.13
	0.500	52.83
	0.250	39.05
	0.125	26.47
	0.062	15.82

Table 3.29 Free radical scavenging activity of isolated compounds on DPPH (0.25 mM) (continues)

Sample	Conc. (mM)	% radical scavenging
Compound **7**	1.000	5.59
	0.500	3.40
	0.250	2.26
	0.125	0.07
Compound **8**	1.000	90.75
	0.500	88.78
	0.250	82.68
	0.125	76.37
	0.062	41.12
Compound **9**	1.000	6.03
	0.500	3.00
	0.250	3.20
	0.125	0.31
Compound **10**	1.000	15.04
	0.500	9.66
	0.250	6.09
	0.125	5.13
Compound **11**	1.000	24.69
	0.500	21.23
	0.250	17.09
	0.125	12.97
Compound **12**	1.000	33.40
	0.500	24.31
	0.250	17.09
	0.125	11.29
Compound **13**	1.000	20.52
	0.500	12.10
	0.250	8.45
	0.125	5.29

Table 3.30 Free radical scavenging activity of BHA on DPPH (0.25 mM)

Sample	Conc. (mM)	% radical scavenging
BHA (Standard)	1.000	90.59
	0.500	87.12
	0.250	73.69
	0.125	57.71
	0.062	25.51
	0.032	7.69

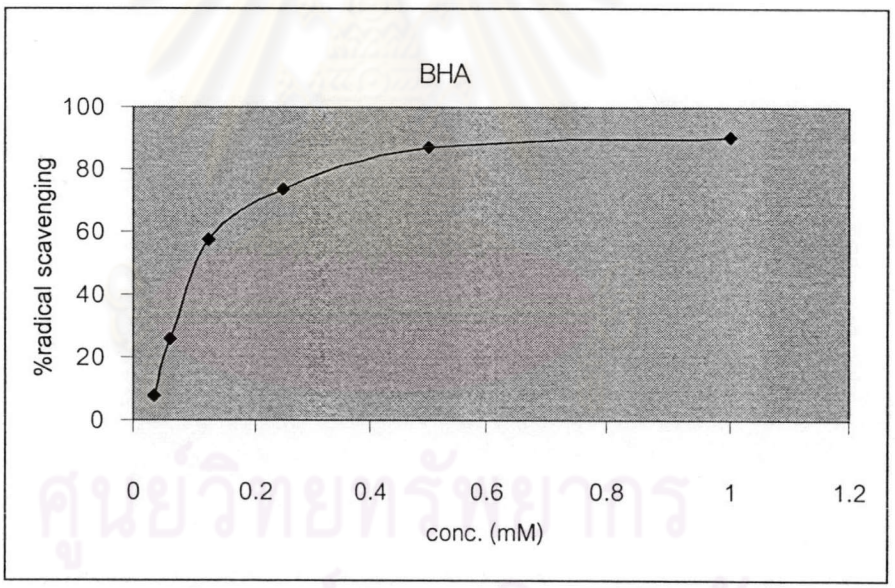


Figure 3.81 Scavenging effect of BHA

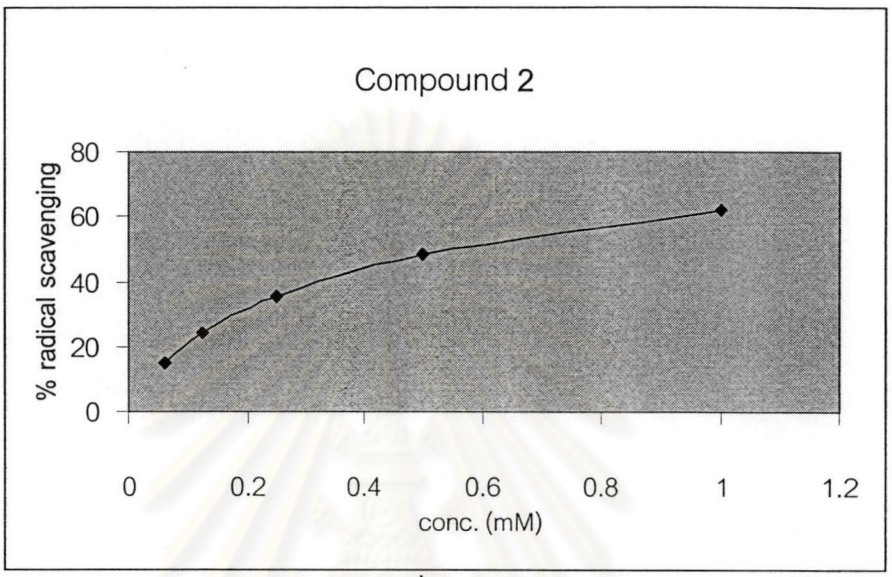


Figure 3.81 Scavenging effect of compound **2**

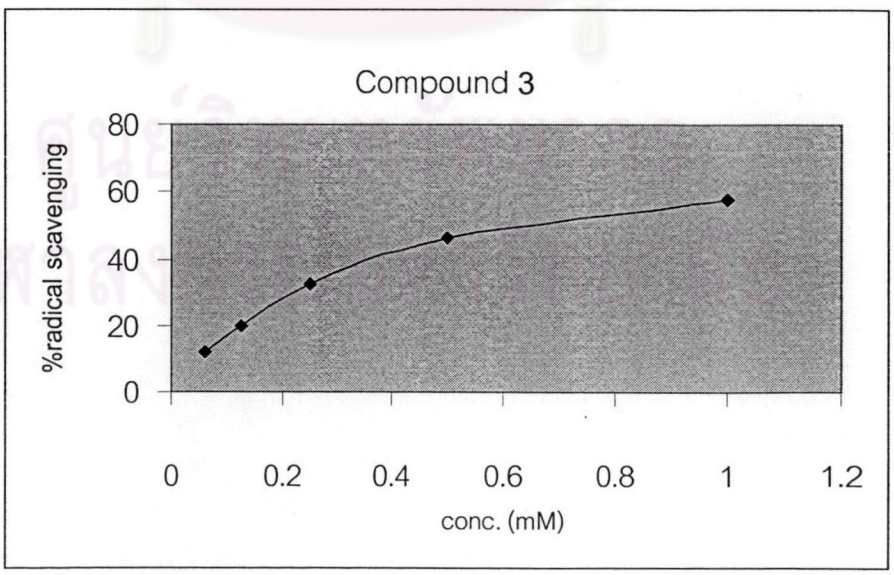


Figure 3.83 Scavenging effect of compound **3**

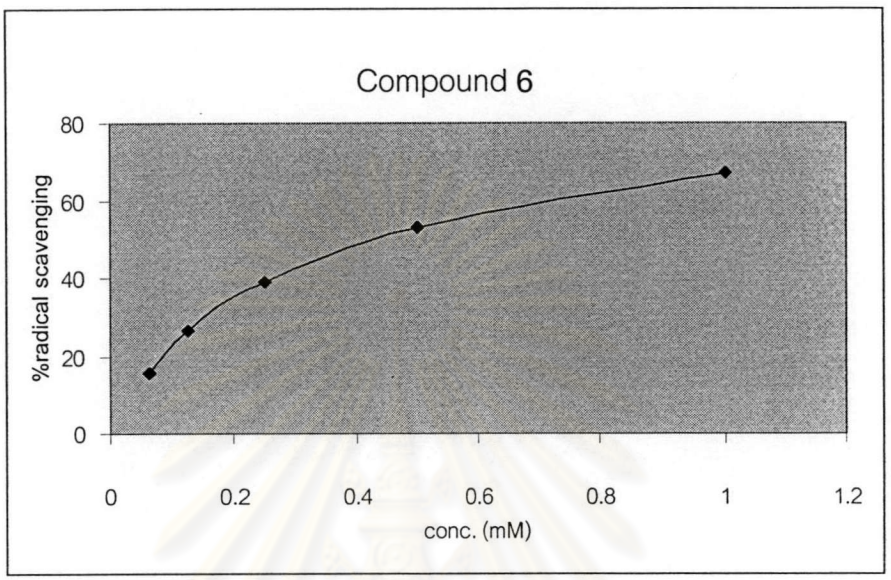


Figure 3.84 Scavenging effect of compound **6**

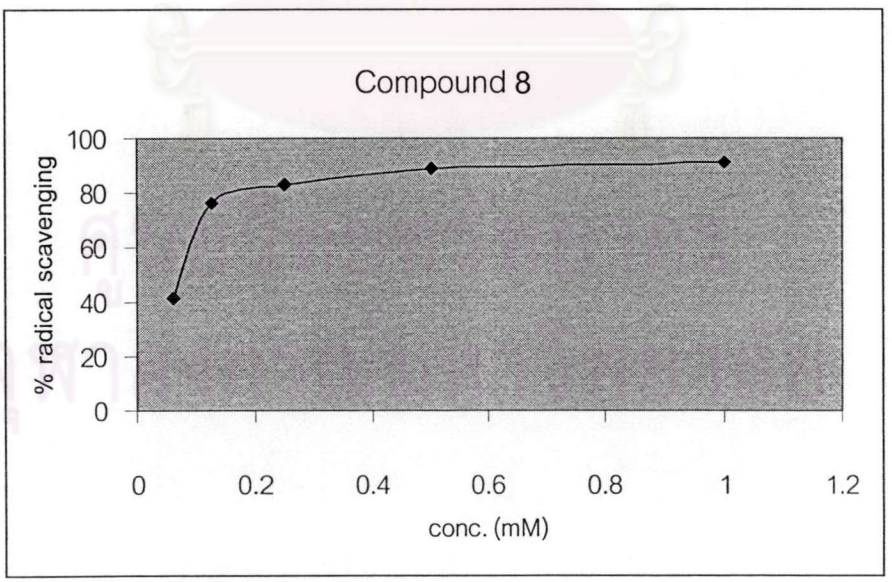


Figure 3.85 Scavenging effect of compound **8**

Table 3.31 IC_{50} on free radical scavenging activity of isolated compound on DPPH

Compound	IC_{50} (mM)
1	> 1.000
2	0.530
3	0.620
4	>1.000
5	> 1.000
6	0.415
7	> 1.000
8	0.080
9	> 1.000
10	> 1.000
11	> 1.000
12	> 1.000
13	> 1.000
BHA	0.120

Table 3.32 Superoxide anion scavenging activity of isolated compounds

Sample	Conc. (μM)	% SOD activity
Compound **1**	100	41.77
	10	35.10
	1	25.27
Compound **2**	100	34.92
	10	32.38
	1	28.01
Compound **3**	100	63.67
	10	51.63
	1	7.66
Compound **4**	100	89.47
	10	23.85
	1	15.21
Compound **5**	100	44.13
	10	29.18
	1	15.40
Compound **6**	100	24.03
	10	21.75
	1	7.26
Compound **7**	100	40.49
	10	32.73
	1	19.62
Compound **8**	100	41.50
	10	24.75
	1	14.81
Compound **9**	100	52.56
	10	13.87
	1	10.60

Table 3.32 Superoxide anion scavenging activity of isolated compounds (continues)

Compound **10**	100	52.29
	10	29.33
	1	15.85
Compound **11**	100	22.76
	10	14.38
	1	2.94
Compound **12**	100	100
	10	90.40
	1	40.88
Compound **13**	100	100
	10	93.71
	1	33.75
BHA	100	100
	10	52.67
	1	17.22

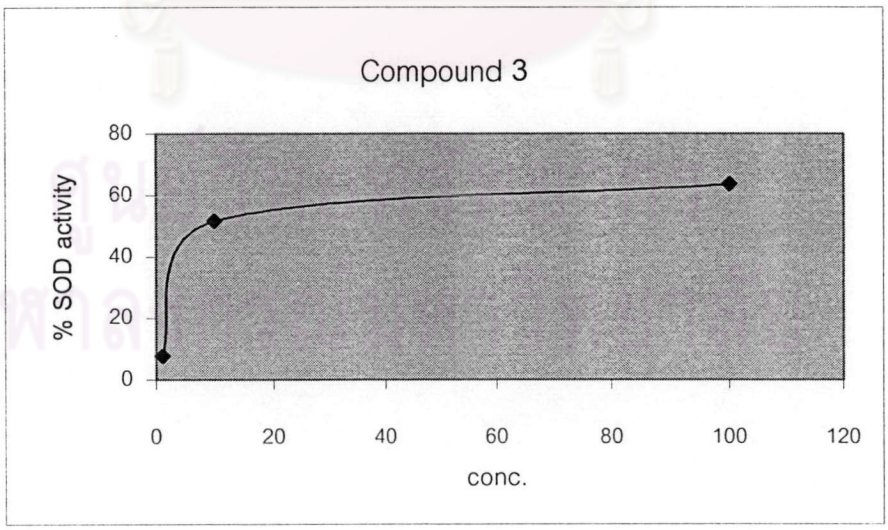


Figure 3.86 Superoxide anion scavenging activity of compound **3**

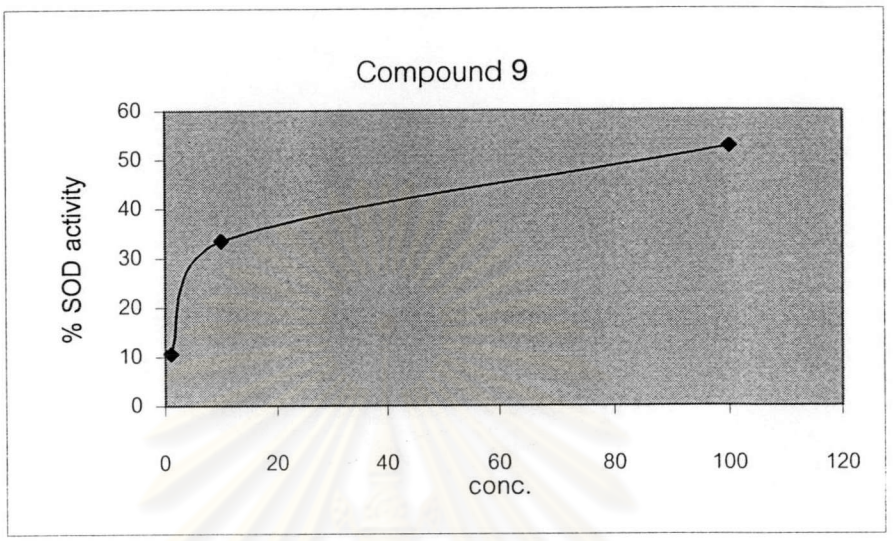


Figure 3.87 Superoxide anion scavenging activity of compound **9**

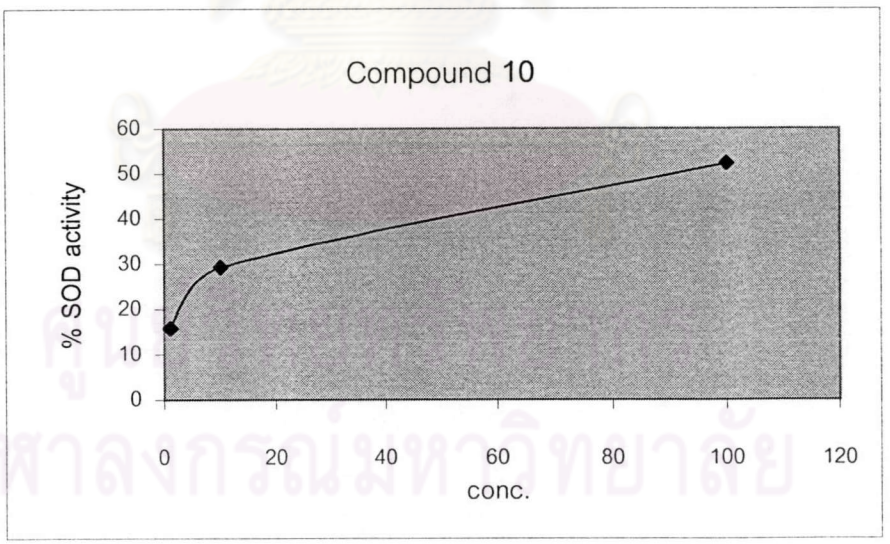


Figure 3.88 Superoxide anion scavenging activity of compound **10**

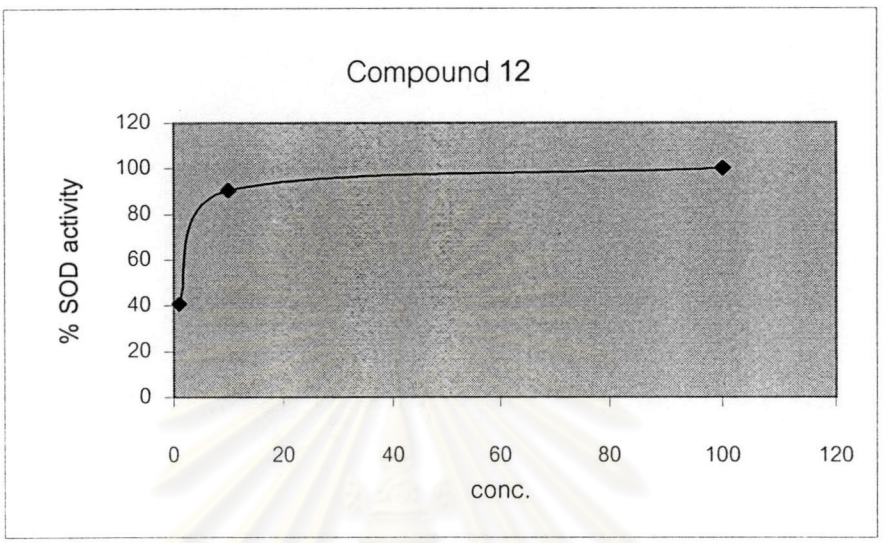


Figure 3.89 Superoxide anion scavenging activity of compound **12**

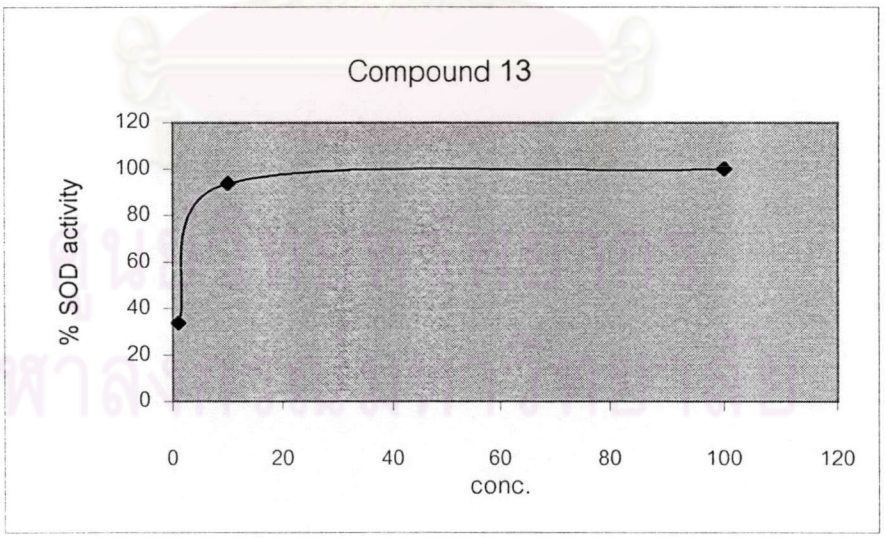


Figure 3.90 Superoxide anion scavenging activity of compound **13**

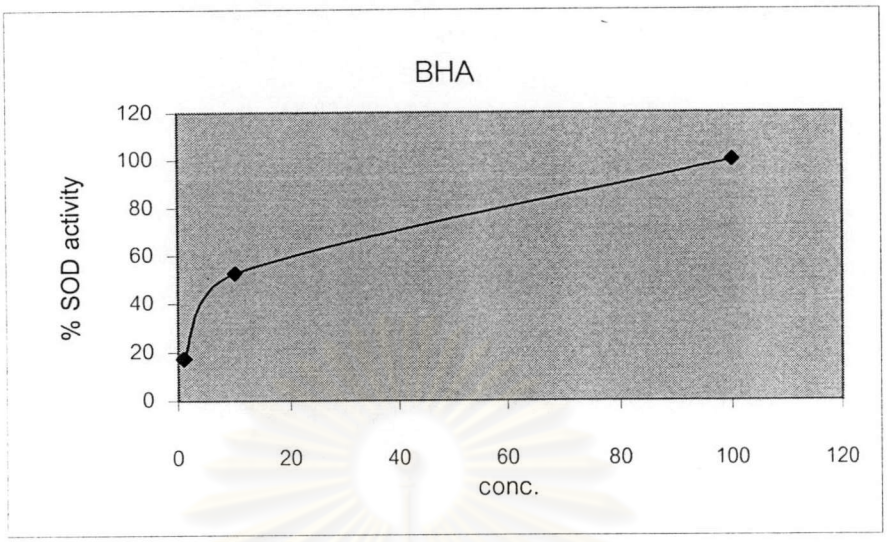


Figure 3.91 Superoxide anion scavenging activity of BHA

Table 3.33 IC_{50} of superoxide anion scavenging activity of isolated compound

Compound	IC_{50} (μM)
1	> 100
2	> 100
3	8.00
4	36.50
5	> 100
6	> 100
7	> 100
8	> 100
9	85.25
10	90.00
11	> 100
12	1.55
13	1.40
BHA	9.50

The isolated compounds fell into six groups. They comprised isoflavone, flavanone, isoflavanone, flavane, chalcone and isoflavone quinone for the last group. The bioassay results of isolated compound were summarized in **Table 3.28, 3.31** and **3.33**.

Cytotoxic activity against KB cell lines of compounds 1-13 by MTT assay.

Among compounds **1-13**, compound **5** and **11** showed moderate in vitro cytotoxicity against KB cell lines (IC_{50} 9 and 8 μg / ml). However, the other compounds were either inactive or weakly active against KB tumor cells.

Free radicals scavenging activity of compounds 1-13.

The free radical scavenging activity of isolated compounds was assessed by DPPH assay. In **Table 3.31**, compound **8** was found to be more potent antioxidant (IC_{50} 0.080 mM) than BHA (IC_{50} 0.120 mM, standard antioxidant), whereas compounds **2**, **3** and **6** showed moderate activity (IC_{50} 0.530, 0.620 and 0.415 mM, respectively).

Superoxide onion scavenging activity of compounds 1-13.

From the above data as shown in **Table 3.33**, It suggested that compounds **3**, **12** and **13** exhibited the strong scavenging effect on $O_2^{\cdot-}$ (IC_{50} 8, 1.55 and 1.40 μM, respectively) which compared with BHA (IC_{50} 9.50 μM). Compound **4** showed moderate superoxide scavenging effect (IC_{50} 36.50 μM) and the other compounds did not show superoxide anion scavenging activity up to the highest concentration (IC_{50} >100 μM).

From above data, the biological activity provide that the free radical and superoxide anion radical scavenging depend on their structures and substituted on B ring. Especially for superoxide anion radicals, the quinone on B ring with the 4-oxo group (compound 11 and 13) showed higher activity than BHA.

From the literature review, the major determinations for radical scavenging capacity are the presence of a catachol group in B ring, which has the better electron-donating properties and is a radical target.

Our data also showed some support of this approach. All of isolated compounds showed no cytotoxicity to living cells so all of them could be developed for more application in pharmaceutical and medicinal areas for the further.