CHAPTER 3

RESULTS AND DISCUSSION

This research has aimed at finding free radical scavengers and structure elucidation of isolated compounds from the barks of *C. brachiata*. This plant was selected for further examination due to the activity of crude extracts on DPPH radical.

Table 3.1 Free radical scavenging test base on DPPH (TLC autographic assay) of various crude extracts

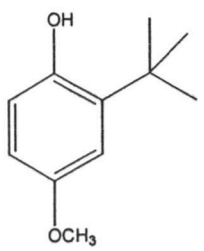
Crude extract	Result
Hexane	Negative
Dichloromethane	Positive
Ethyl acetate	Positive
Methanol	Positive

Crude extracts that showed the positive results were further tested with spectrophotometric assay.

Table 3.2 IC_{50} of crude extracts against DPPH radical comparison with BHA and Vitamin E

Sample	IC_{50} (ppm)
Crude Dichloromethane	951.02
Crude Ethyl acetate	222.23
Crude Methanol	110.81
Vitamin E	74.49
BHA*	16.88

* BHA : butylated hydroxyanisole



3.1 Properties and Structure Elucidation of Isolated Mixtures and Compounds

3.1.1 Mixtures 1

This mixture was obtained from dichloromethane crude extract by eluting with CH_2Cl_2/hexane (1:9 to 3:7) on column chromatography and was recrystallized from CH_2Cl_2/hexane (1:4) to afford 65 mg of white powder. Its melting point was 50-63 °C, a broad range of melting point, could indicate that this powder was a mixture.

The IR spectrum **(Figure 3.1)** showed strong absorption bands at ν_{max} 2909, 2843 (C-H stretching of aliphatic) and 1706 cm^{-1} (C=O stretching of ester). From the melting point range and IR data it could be concluded that the white powder should be a mixture of long chain aliphatic ester.

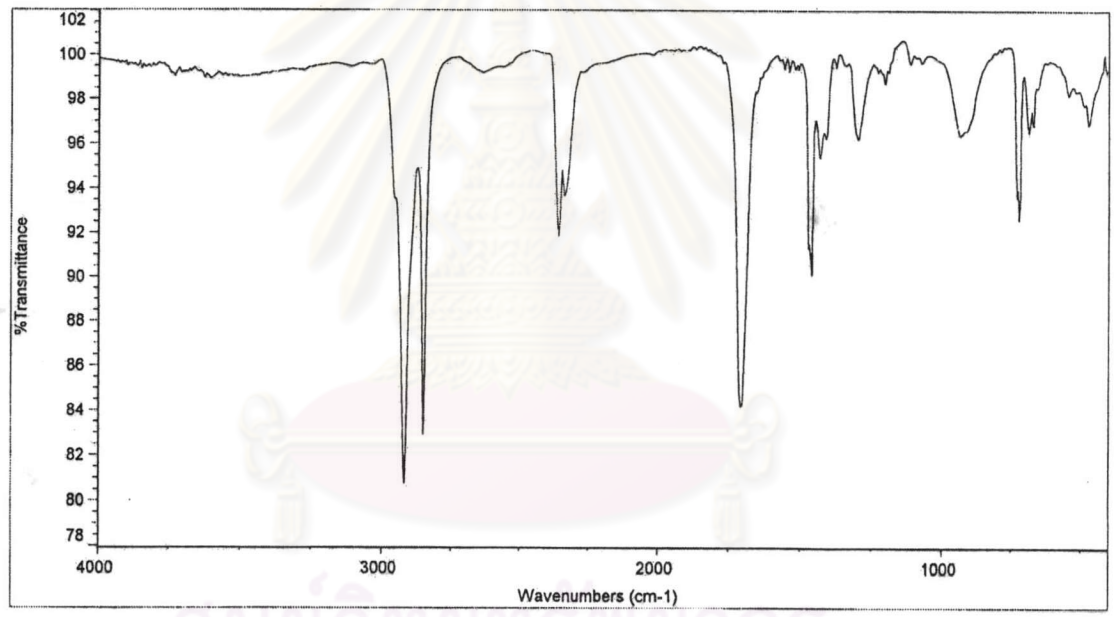


Figure 3.1 IR spectrum of mixture **1**

3.1.2 Mixture 2

Dichloromethane crude extract was separated by open column chromatography and recrystallized with CH_2Cl_2/hexane (1:1) to afford 20 mg of colorless needle, mixture **2**. It cannot be detected on TLC under UV light, but can be detected with 10% H_2SO_4 in ethanol. This compound was indicated that no a conjugated double bond in the molecule. Its melting point was 148-150 °C.

From the melting point range and polarity, it could be suggested that the colorless needle is a mixture of β-sitosterol and stigmasterol, the common steroid that found in plants. GC technique was used to confirm this suggestion, by comparison with an authentic steroid.

The GC spectrum **(Figure 3.2)** (OV-1 column, injection temperature 300 °C, column temperature 260 °C, carrier gas N_2 flow rate 50ml/min, FID detector) gave two peaks with retention time of 25.63 and 28.97 minutes which corresponded to those of an authentic stigmasterol **(Figure 3.3)** and β-sitosterol **(Figure 3.4)**, respectively. Then it could be decided that the Mixture **2** consisted of stigmasterol and β-sitosterol.

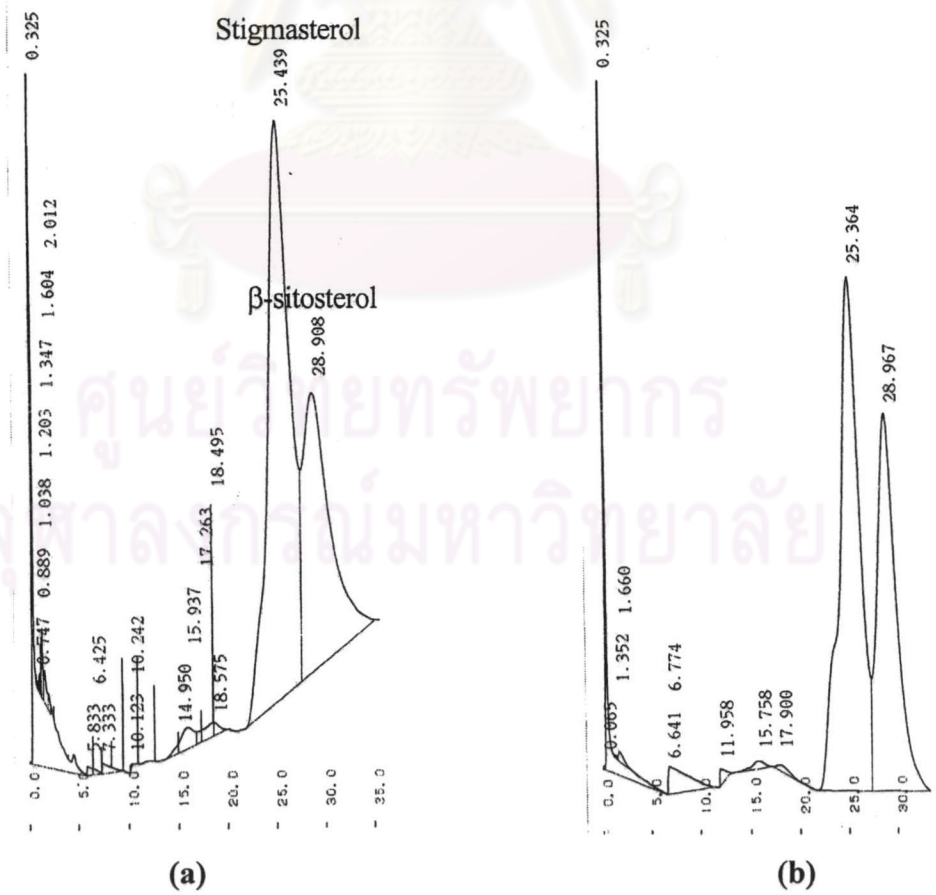


Figure 3.2 Chromatograms of (a) standard steroids and (b) mixture **2**

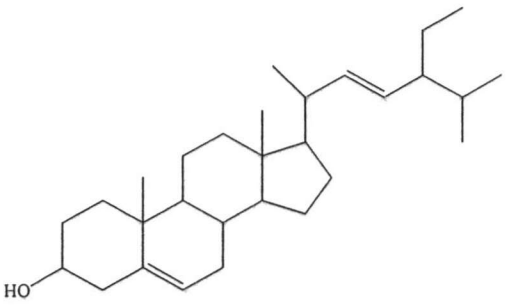


Figure 3.3 Structure of stigmasterol

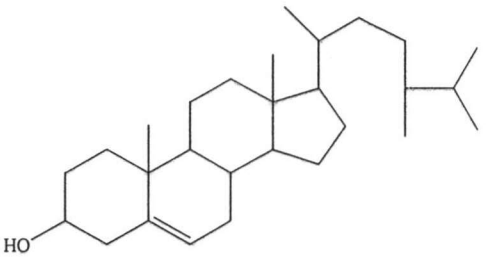


Figure 3.4 Structure of β-sitosterol

3.1.3 Mixture 3

The white powder (78 mg) was obtained from dichloromethane crude extract by eluting with CH_2Cl_2/hexane (3:7 to 7:3) and repeated column chromatography by using CH_2Cl_2/hexane (5:6) as a mobile phase. Its spot on TLC can be detected under UV light, so it was further purified with chromatotron by eluting with EtOAc/hexane (1:9).

Its melting point was 68-70 °C (lit[32] 68-71 °C). The IR spectrum **(Figure 3.5)** indicated the presence of a hydroxyl group at ν_{max} 3506 cm^{-1} and a conjugated ester at 1724 cm^{-1} besides, the characteristic absorption peak due to an aromatic moiety was observed at 1640, 1598, 1521 and 1465 cm^{-1}.

The ^1H NMR ($CDCl_3$) spectrum **(Figure 3.6)** exhibited the characteristics of a feruloyl moiety a methoxy singlet at δ 3.91 (3H,s), a hydroxyl singlet at δ 5.85 (1H,s), two trans olefinic protons δ 6.27 (1H, d, J = 16.1 Hz) and 7.59 (1H, d, J = 15.9 Hz) and three aromatic protons in a 1,3,4-trisubstitution pattern at δ 6.90 (1H, d, J = 8.1 Hz), 7.02 (1H, d, J = 1.6 Hz) and 7.05 (1H, d, J = 8.2 Hz) due to H-5′, H-2′ and H-6′, respectively. Furthermore an aliphatic moiety was observed by a signal at δ 0.86 (3H, t, J = 6.0 Hz, terminal methyl), a number of methylene protons at δ 1.25, a quintet signal at δ 1.68 (2H, quint, J = 6.9 Hz) for methylene next to the downfield methylene and the deshielded triplet at δ 4.17 (2H, t, J = 6.7 Hz) corresponding to a methylene adjacent to an oxycarbonyl functionallity.

The ^{13}C NMR ($CDCl_3$) and DEPT 90, 135 spectrum **(Figure 3.7 – 3.9)** also supported the presence of a ferulate ester with resonances attributable to a carbonyl group (δ 167.4), two deshielded oxygen bearing quarternary carbons (δ 146.7 and

147.9), five methine carbons (δ 109.3, 114.7, 115.7, 123.0 and 144.6), and a shielded aromatic quaternary carbon (δ 127.0). The assignment of the ^{13}C NMR **(Table 3.3)** chemical shifts was achieved by comparison with those of reported compounds.[32]

The EI mass spectrum **(Figure 3.10)** showed molecular ion peaks at m/z 586, 572, 558, 544 and 530 corresponding respectively to ferulate ester molecule ions with the alkyl portions of $C_{24}H_{49}$, $C_{25}H_{51}$, $C_{26}H_{53}$, $C_{27}H_{55}$ and $C_{28}H_{57}$. The fragments **(Scheme 3.1)** at m/z 194 and 177 corresponded to ferulic acid and feruloyl fragments, respectively. Mixture **3** was therefore a mixture of five alkyl *trans*-ferulate bearing alkyl groups of C_{24} to C_{28}. **(Figure 3.11)**

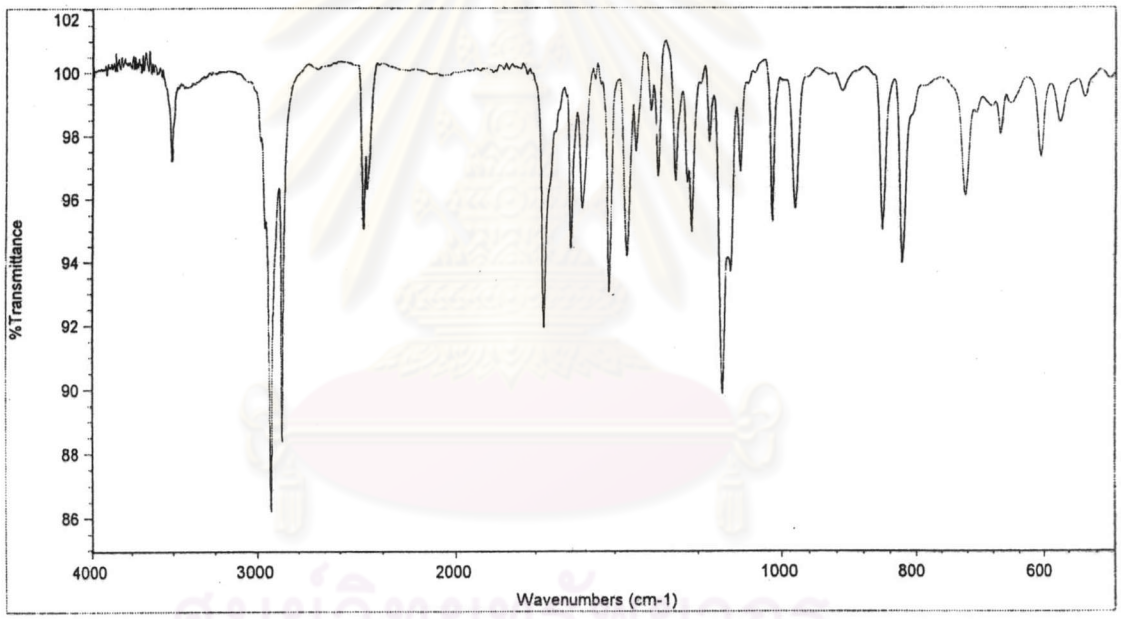


Figure 3.5 IR spectrum of mixture **3**

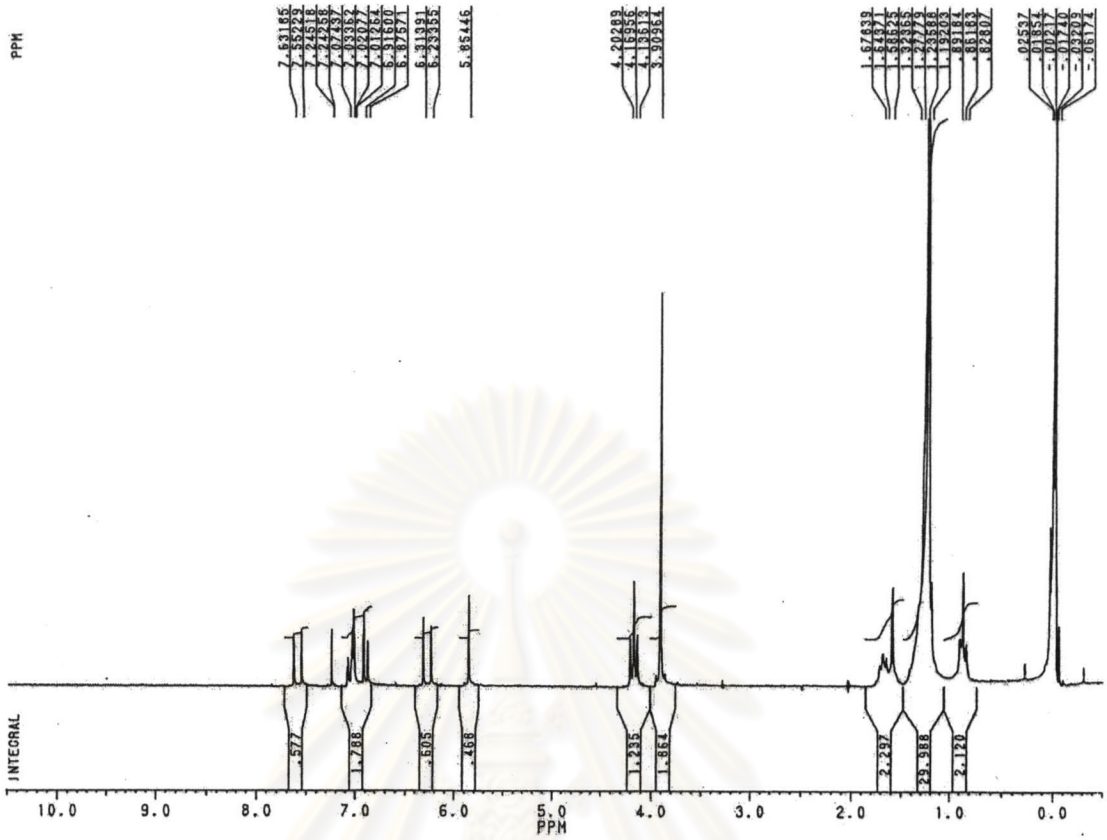


Figure 3.6 ^{1}H NMR spectrum of mixture **3**

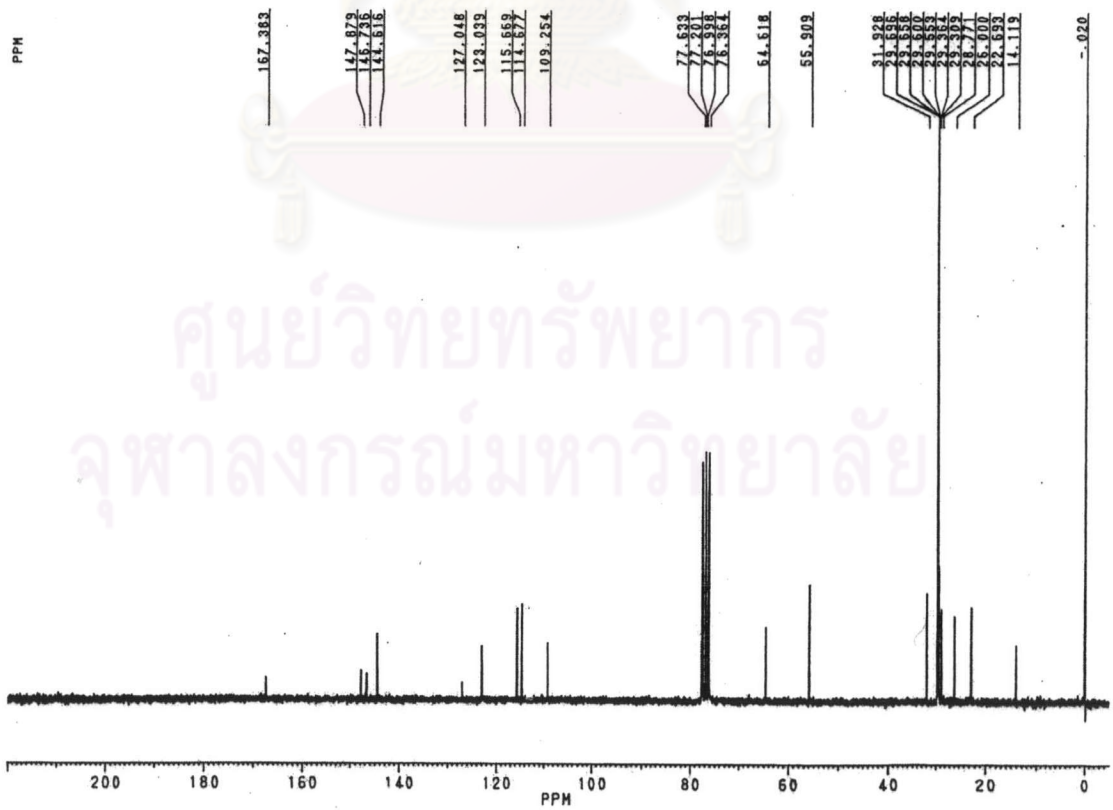


Figure 3.7 ^{13}C NMR spectrum of mixture **3**

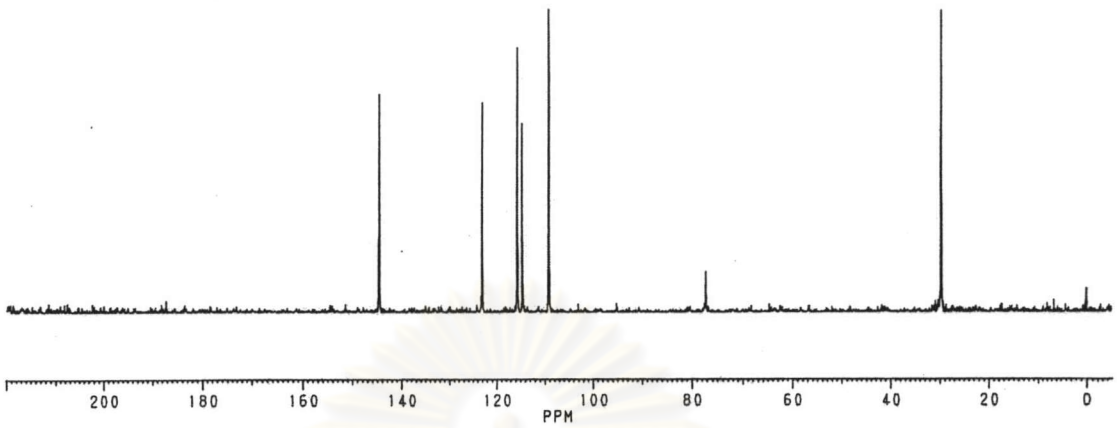


Figure 3.8 DEPT 90 spectrum of mixture **3**

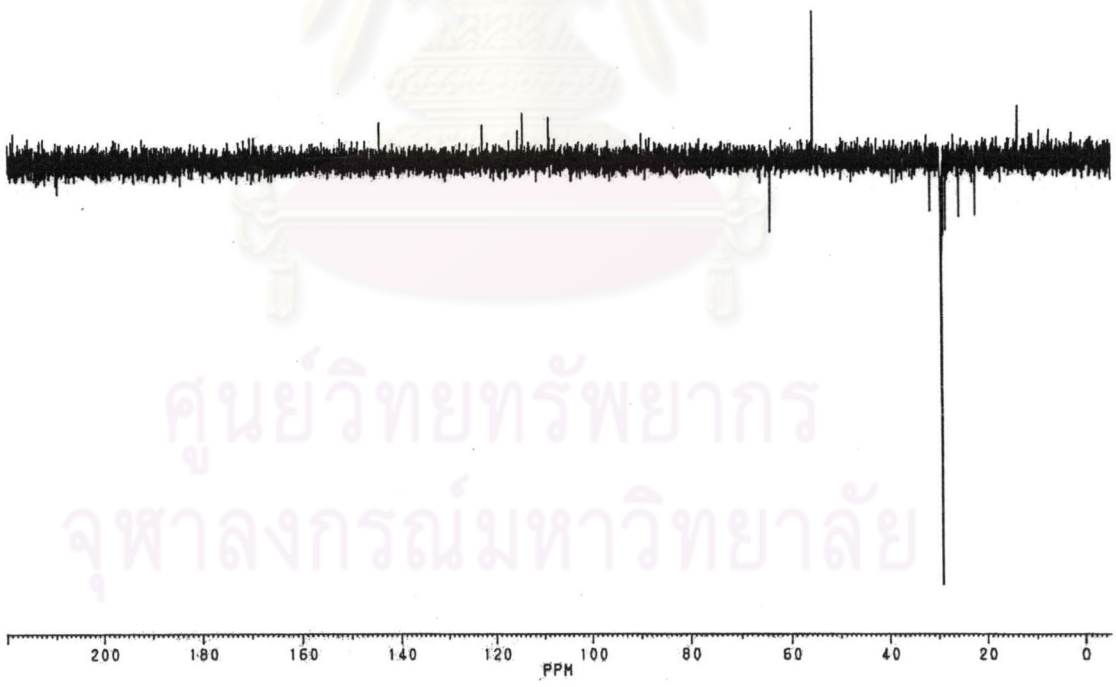


Figure 3.9 DEPT 135 spectrum of mixture **3**

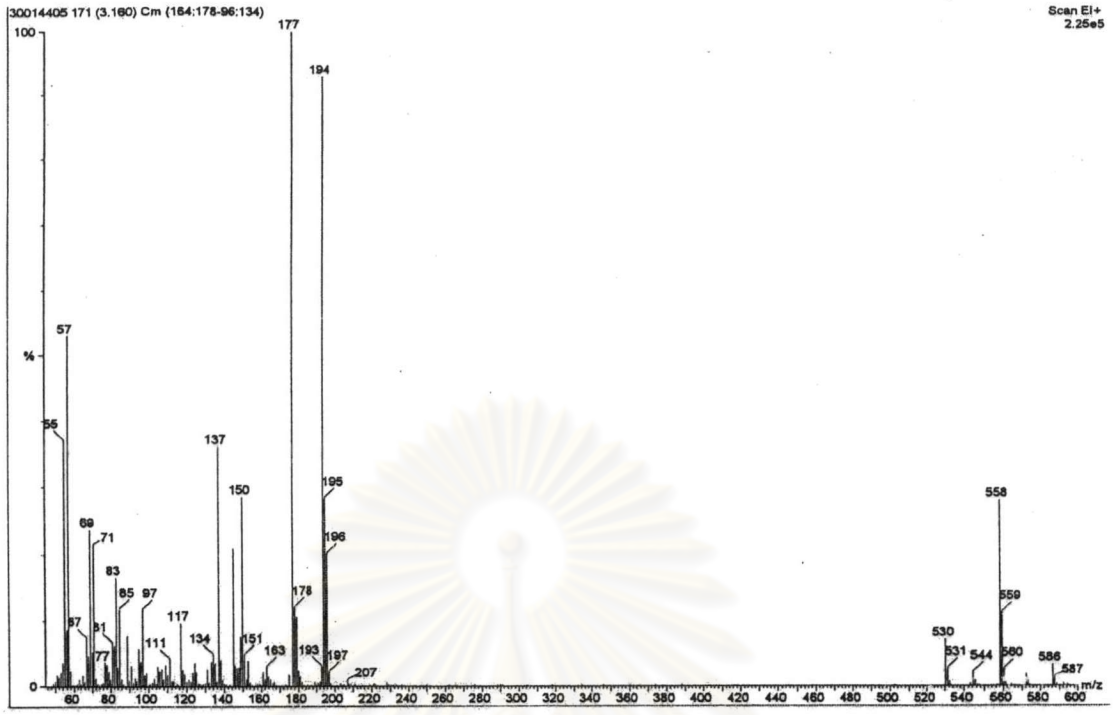
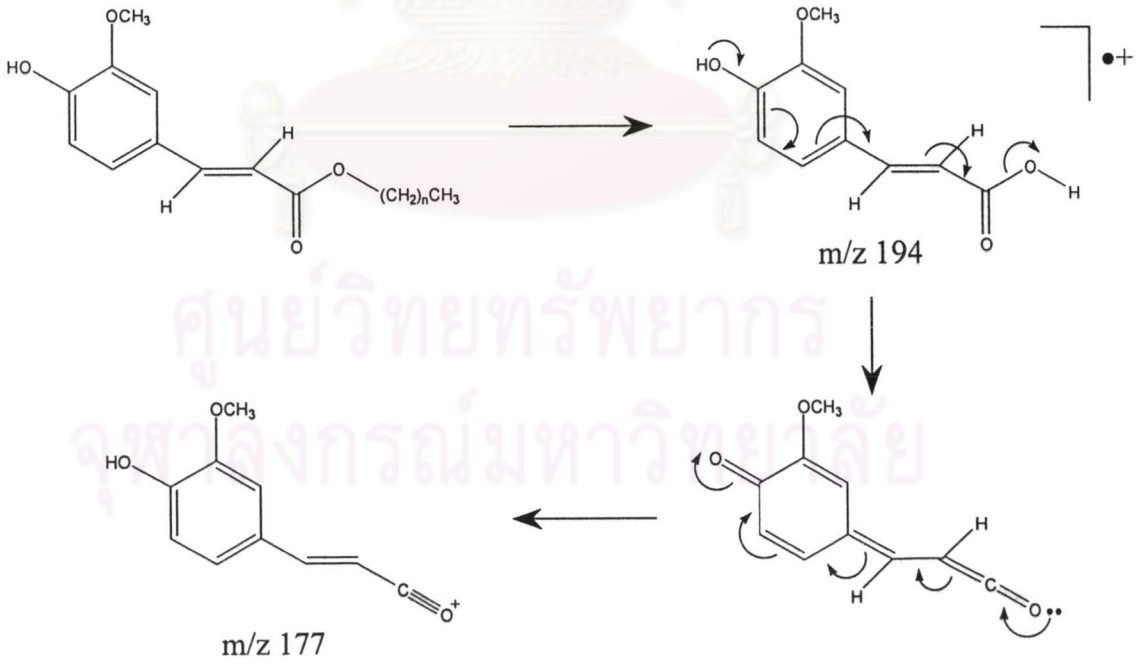
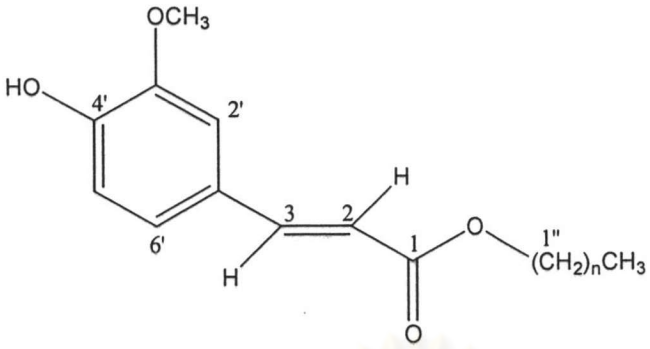


Figure 3.10 EIMS spectrum of mixture **3**



Scheme 3.1 Mass fragmentation pattern of mixture **3**



n = 23 - 27

Figure 3.11 Molecular structure of mixture **3**

Table 3.3 ^{13}C NMR data of mixture **3** compared with alkyl *trans*-ferulate

position	Mixture **3**	Alkyl *trans*-ferulate
1	167.4	167.8
2	115.7	116.1
3	144.6	145.0
1'	127.0	127.5
2'	109.3	109.7
3'	147.9	148.3
4'	146.7	147.1
5'	114.7	115.1
6'	123.0	123.5
1"	64.6	65.0
2"	28.8	29.2
3"	26.0	26.4
4" to (n-2)"	29.3 – 29.7	29.7 – 30.1
(n-1)"	31.9	32.3
n"	22.7	23.1
Me	14.1	14.5
OMe	55.9	56.3

3.1.4 Compound 1

Compound **1** (19 mg) was obtained as a white powder of melting point 212 – 214 °C from crude dichloromethane and crude ethyl acetate.

The IR spectrum **(Figure 3.12)** showed very broad absorption bands for hydroxyl at ν_{max} 3500 – 2500 cm^{-1}, carbonyl for carboxylic functional group at 1680 cm^{-1}, and C-O stretching at 1245 cm^{-1}.

The ^{1}H NMR (CD_3OD) spectrum **(Figure 3.13)** exhibited two peak of aromatic protons at δ 6.89 (2H, d, J = 8.54 Hz) and δ 7.87 (2H, d, J = 8.85 Hz). The splitting pattern (distorted doublet) suggested the presence of unequivalent *para*-substituted groups.

The ^{13}C NMR (CD_3OD) spectrum **(Figure 3.14)** showed three quaternary carbon signals resonated at δ 170.1, 163.4, 122.8 and two methine carbon signals at δ 133.0 and 116.3 which in each signal have two atoms of methine carbon.

The EI-MS **(Figure 3.15)** gave peak at m/z 138 as a molecular peak.

All spectral data suggested that compound **1** was *p*-hydroxybenzoic acid. NMR assignment of compound **1** was shown in **Figure 3.16** and was achieved based upon previous literature[33]

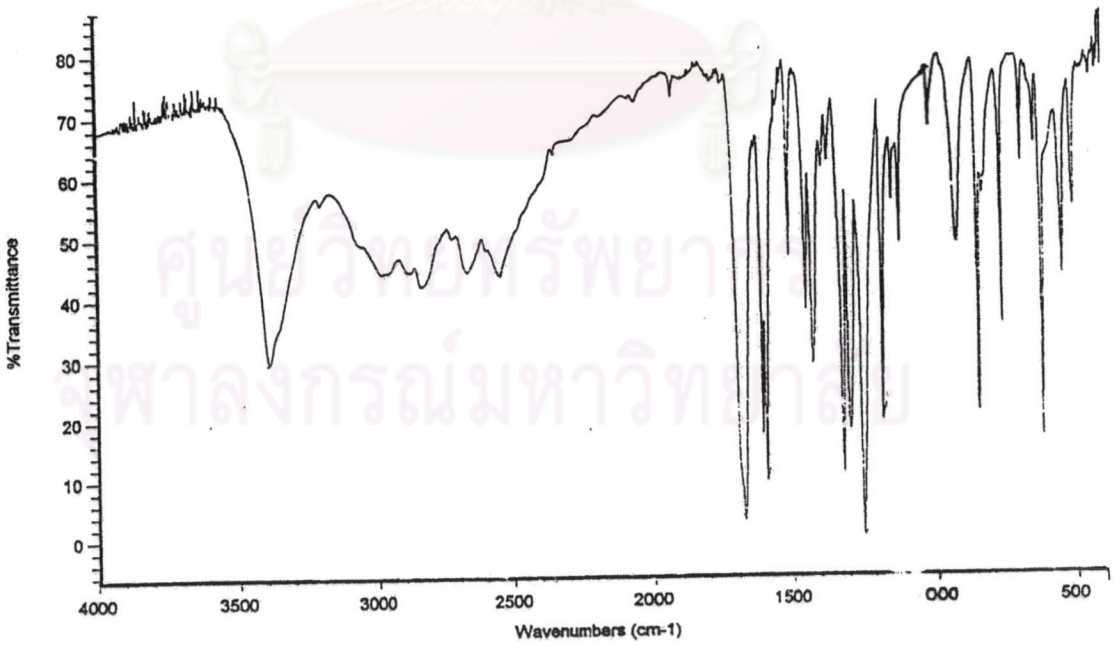


Figure 3.12 IR spectrum of compound **1**

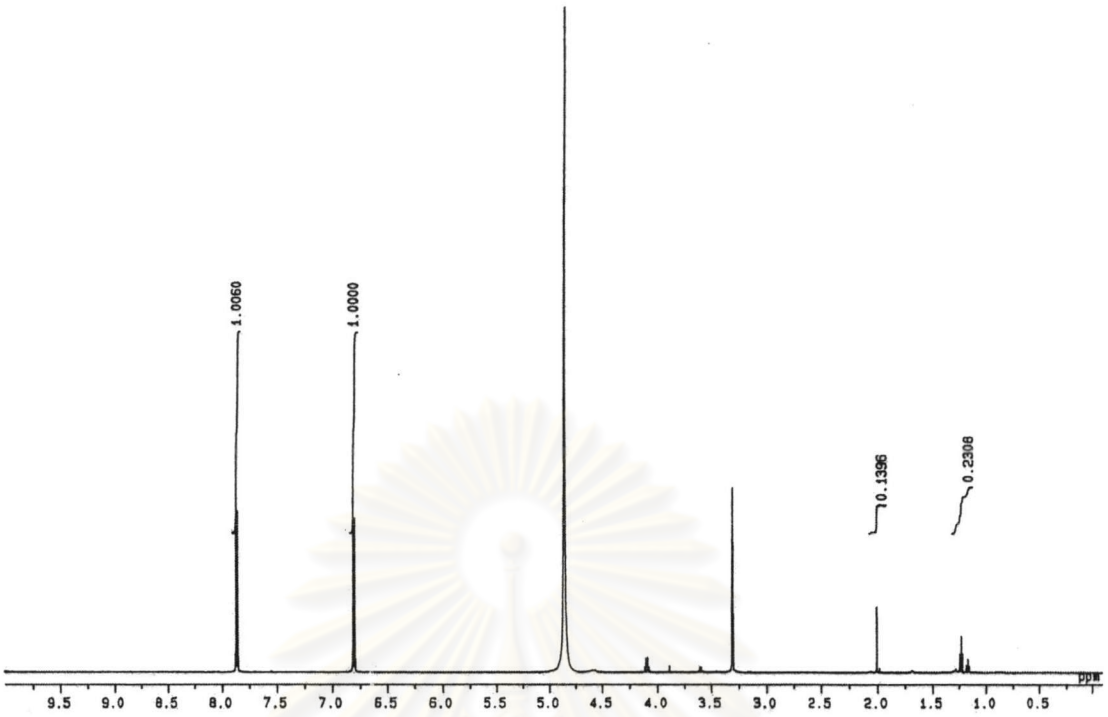


Figure 3.13 ^{1}H NMR spectrum of compound **1**

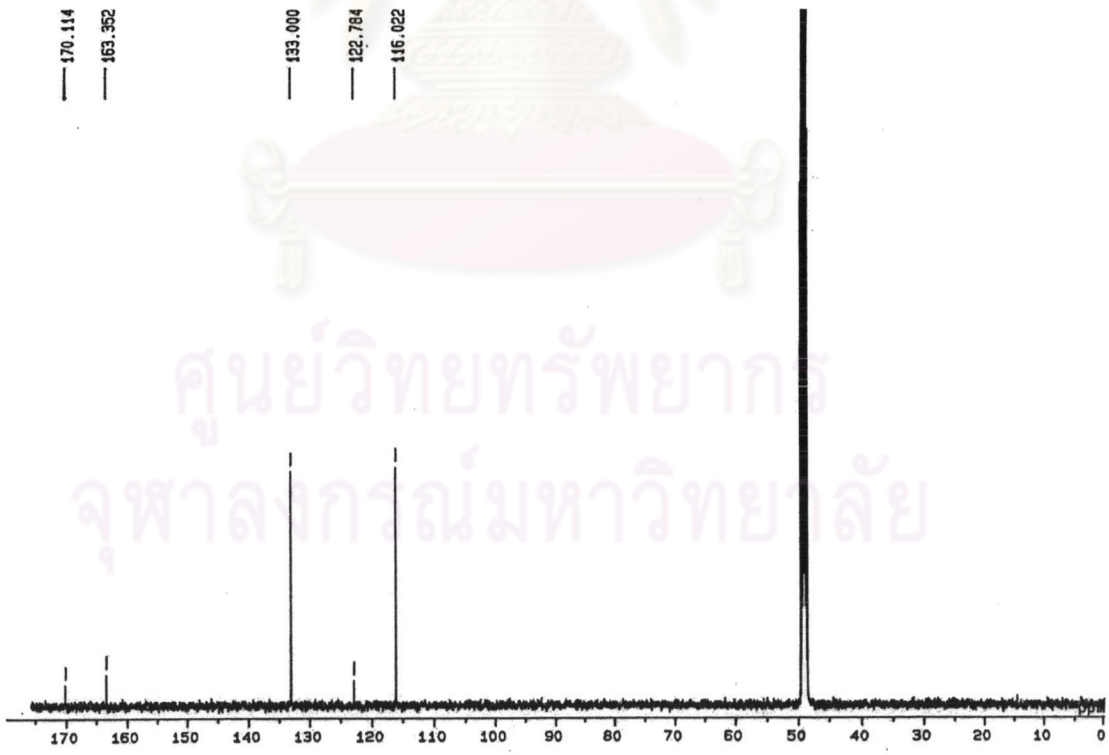


Figure 3.14 ^{13}C NMR spectrum of compound **1**

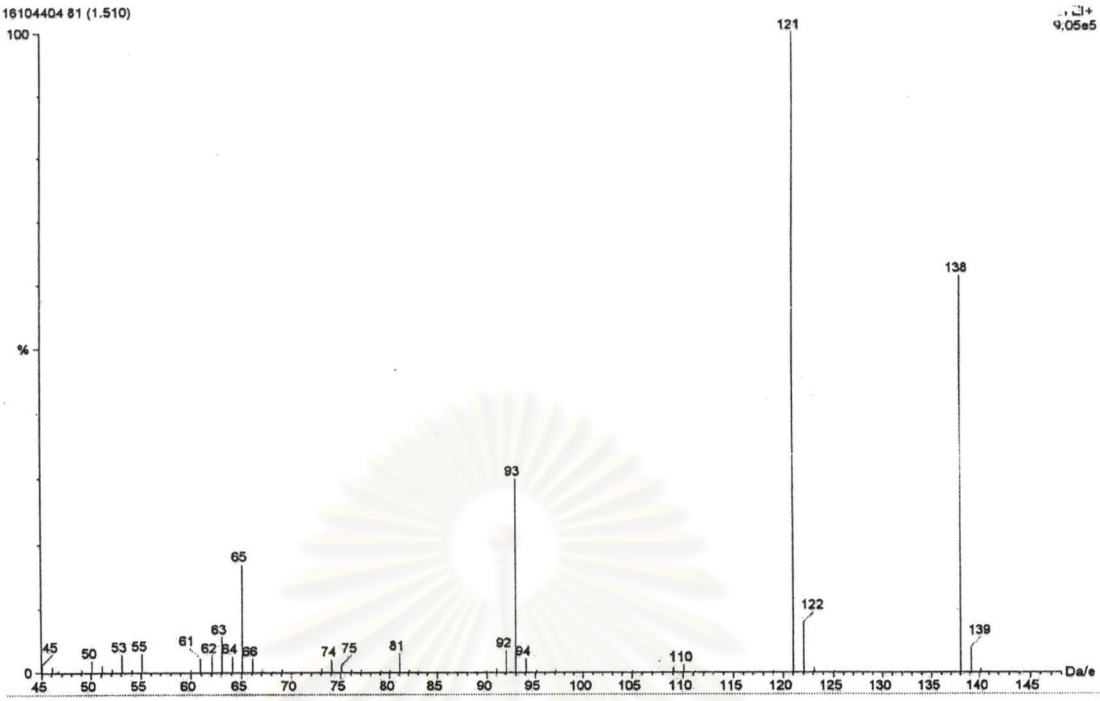


Figure 3.15 EIMS spectrum of compound **1**

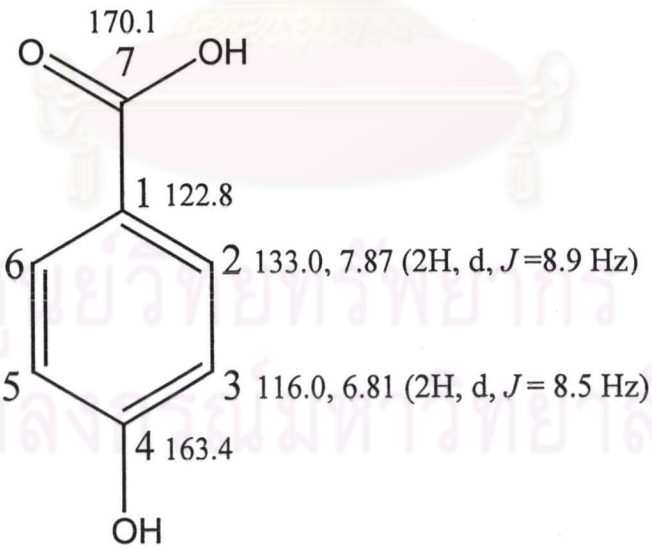


Figure 3.16 Proton and Carbon assignments of compound **1**

The carboxylic and phenolic protons were absence because of their exchangeability with deuterated solvent (CD_3OD).

3.1.5 Compound 2

Compound 2 was obtained as an off white amorphous powder (42 mg) from ethyl acetate crude extract. Activity (DPPH) – directed fractionation of this extract, afforded this compound from silica gel column chromatography (EtOAc/CH_2Cl_2, 4:9 to 4:1). For further purification, this compound was rechromatographed with the gradient between EtOAc and CH_2Cl_2 several times but still could not obtained the pure compound. So we decided to repeat chromatography of this compound by using CH_2Cl_2/ether (8:3) as mobile phase yielded the pure white powder of compound **2** deposited after left for two hours. This compound was decomposed at 246 °C

The IR spectrum **(Figure 3.20)** showed a broad absorption band of polyhydroxyl group at ν_{max} 3375 cm^{-1} and the characteristic absorption band of aromatic moiety was observed at ν_{max} 1619, 1516 and 1455 cm^{-1}.

The NMR spectra coupled with a $[M+H]^+$ peak at m/z 545 from the positive – ion FABMS **(Figure 3.21)** analysis indicated a molecular formula for compound **2** of $C_{30}H_{24}O_{10}$, showing 19 degrees of unsaturation.

The NMR spectra of compound **2** were similar to those of other A-type proanthocyanidin.[34,35] The ^1H NMR spectrum **(Figure 3.22)** were operated by used acetone-d_6 as a solvent. The hydroxyl peak could be observed as a singlet peak (sharp) and some of them showed coupling with the adjacent proton. So, the spectra of this compound had some difference from the A-type proanthocyanidins of those reported in the literature which used methanol-d_4 as a solvent. However, the ^1H NMR spectrum of compound **2** displayed the characteristic signals for H-3 and H-4 protons of an A-type proanthocyanidin at δ 4.25 (1H, dd, J = 3.66, 3.36 Hz) and 4.38 (1H, d, J = 3.36 Hz), respectively. The aromatic region of this compound showed the presence of two *meta*-coupled doublets at δ 5.88 (1H, d, J = 2.44 Hz) and 6.07 (1H, d, J = 2.44 Hz) which indicated the presence of a tetra-substituted benzene ring. On the other hand, a singlet at δ 6.13 (1H, s) was corresponded to penta-substituted benzene ring. The four A_2B_2 – type doublets were observed at δ 6.86 (2H, d, J = 8.55 Hz), 6.89 (2H, d, J = 8.85 Hz), 7.51 (2H, d, J = 8.54 Hz) and 7.56 (2H, d, J = 8.85 Hz), which demonstrated the presence of two *p*-substituted benzene rings. The other protons appeared at δ 8.51 (2H, s), 8.45 (1H, s), 8.18 (1H, s), 7.08 (1H, s), 4.39 (1H, d, J = 4.88 Hz), 4.03 (1H, d, J = 6.19 Hz) were the hydroxyl protons and protons at δ 5.16

(1H, s), 4.30 (1H, m) and 2.90 (2H, dd, J = 4.27, 2.44 Hz) were the protons of another dihydropyran unit. The assignment of proton exhibited in **Table 3.4**

The ^{13}C NMR, DEPT and 2D-NMR techniques were used for supported the assignments (All the spectra are showed in **Figure 3.23 – Figure 3.28**)

The ^{13}C NMR spectrum of compound **2** indicated the presence of 30 carbon atoms (**Table 3.4**), which included 6 aliphatics and 20 aromatics (with 4 of double intensity). The DEPT spectrum showed the absence of CH_3 groups, with the presence of one methylene, 11 methines (four of double intensity) and 14 quaternary carbon atoms. The flavan moieties can be linked to build up the basic skeleton of an A-type proanthocyanidin through three type of linkages, namely (4→8, 2→O→7), (4→6, 2→O→5) and (4→6,2→O→7). A HMBC spectrum which showed long-range coupling of the H-4 proton with C-7' and C-9' **(Figure 3.17)**; thus, defined the (4→8, 2→O→7) linkage. Moreover, it is important to point out that the correlation between H-4 and H-12', H-16' protons from the NOESY spectra **(Figure 3.18)**, provided strong evidence for the C-4/C-8' and C-2→O→C-7' interflavanoid linkages. The next step was then to assign the stereochemistry at the chiral centers. In the case of C-2 and C-4, the absolute stereochemistry could not be determined by NMR data. The appearance of a singlet at δ 5.16 due to H-2' suggested the presence of 2',3'-*cis* (epiafzelechin type) stereochemistry. This was supported by comparison with the known compound.[36] The last chiral carbon (C-3), due to NOESY experiment showed the weak correlation of hydroxyl group on C-3 to H-12, H-16 and very weak to H-13, H-15. According to this NOESY data, we could be assigned the hydroxyl on C-3 was in another plane of phenyl group at C-2 (afzelechin type). On the basis of all these observations, the possible structures of compound **2** were proposed to be afzelechin-(4α→8, 2α→ O→7)-epiafzelechin **(2a)** or afzelechin-(4β→8, 2β→O→7)-epi afzelechin **(2b) Figure 3.19**. In general method for complete assignment of absolute stereochemistry, circular dichroism (CD) measurement will be used.

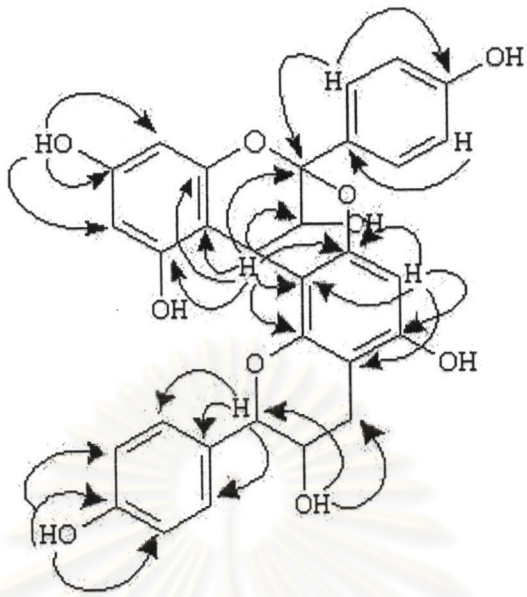


Figure 3.17 Selected HMBC correlation of compound **2**

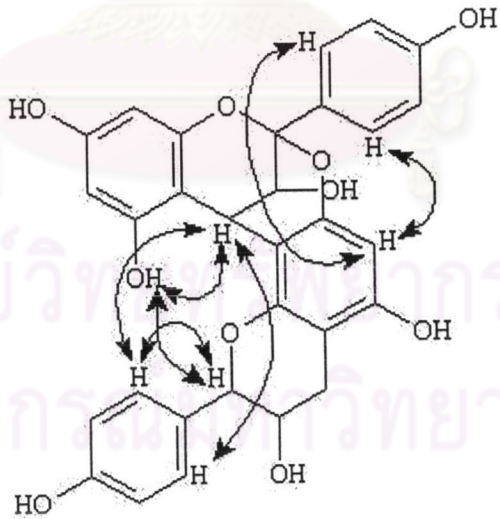
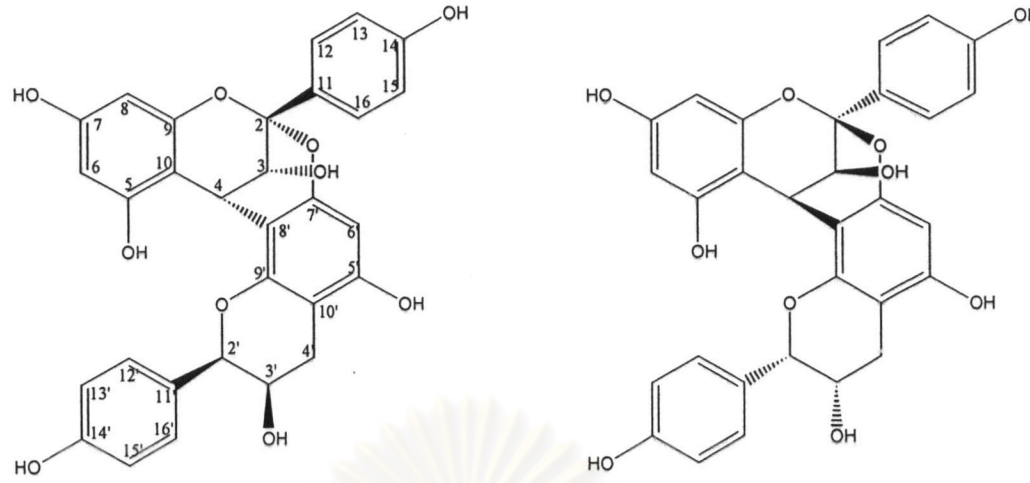


Figure 3.18 Selected NOESY correlation of compound **2**



2a **2b**

Figure 3.19 Two possible structures of compound **2**

Table 3.4 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **2**[a]

Position	δH (*J* in Hz)	δC	DEPT	^{1}H-^{13}C connectivities (HMBC)
2		100.2	C	
3	4.25 (1H, dd, *J* = 3.66, 3.36)	67.1	CH	100.2, 103.8, 106.5
4	4.38 (1H, d, *J* = 3.36)	28.8	CH	67.1, 100.2, 103.8, 106.5, 150.9, 151.6, 156.5, 154.0
5		156.5	C	
6	5.88 (1H, d, *J* = 2.44)	97.6	CH	96.1, 103.8
7		157.9	C	
8	6.07 (1H, d, *J* = 2.44)	96.1	CH	97.6, 103.8, 154.0, 157.9
9		154.0	C	
10		103.8	C	
11		131.5	C	
12, 16	7.56 (2H, d, *J* = 8.85)	129.4	CH	100.2, 129.4, 158.5
13, 15	6.86 (2H, d, *J* = 8.55)	115.2	CH	115.2, 131.5, 158.5
14		158.5	C	
2'	5.16 (1H, s)	80.6	CH	129.1, 130.4

Table 3.4 (cont.) ^{1}H NMR (500 MHz) and ^{13}C nmr (125 MHz) data of compound **2**[a]

Position	δH (*J* in Hz)	δC	DEPT	^{1}H-^{13}C connectivities (HMBC)
3'	4.30 (1H, m)	66.5	CH	29.3
4'	2.90 (2H, dd, *J* =4.27, 2.44)	29.3	CH_2	66.5, 80.6, 101.7
5'		156.0	C	
6'	6.13 (1H, s)	96.3	CH	101.7, 106.5, 151.6, 156.0
7'		151.6	C	
8'		106.5	C	
9'		150.9	C	
10'		101.7	C	
11'		130.4	C	
12', 16'	7.51 (2H, d, *J* = 8.54)	129.1	CH	80.6, 129.1, 158.1
13', 15'	6.89 (2H, d, *J* = 8.85)	115.8	CH	115.8, 130.4, 158.1
14'		158.1	C	
OH-3	4.39 (1H, d, *J* = 4.88)			28.8, 67.1, 100.2
OH-5	7.08 (1H, s)			97.6, 103.8, 156.5
OH-7	8.18 (1H, s)			96.1, 97.6, 157.9
OH-14, OH-5'	8.51 (2H, s)			96.3, 101.7, 115.2, 156.0, 158.5
OH-3'	4.03 (1H, d, *J* = 6.19)			29.3, 66.5, 80.6
OH-14'	8.45 (1H, s)			115.8, 158.1

[a] Measured in Me_2CO-d_6

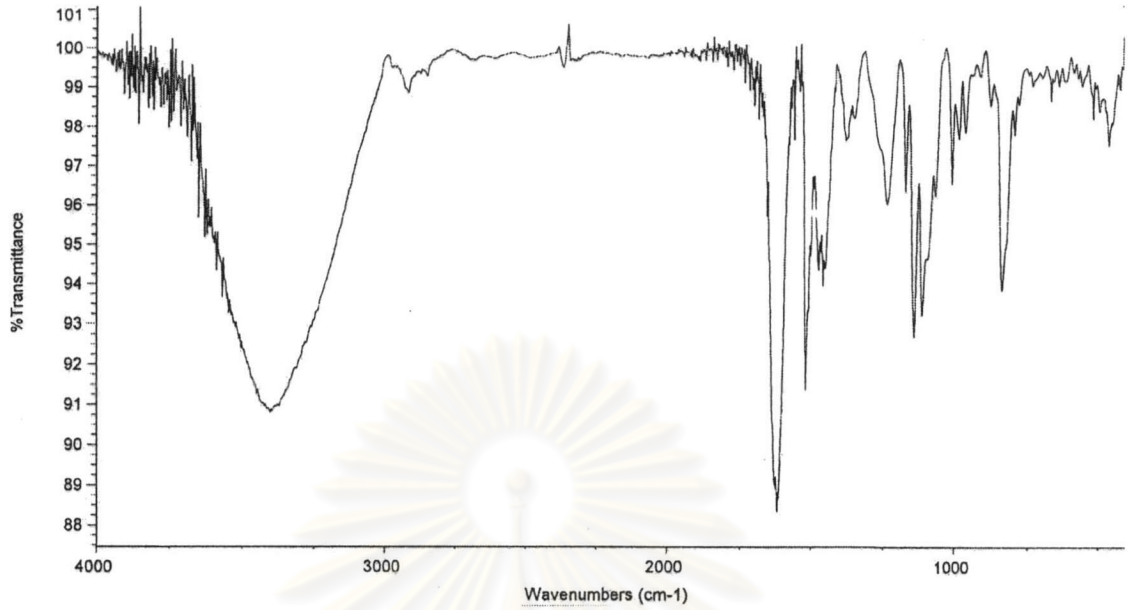


Figure 3.20 IR spectrum of compound **2**

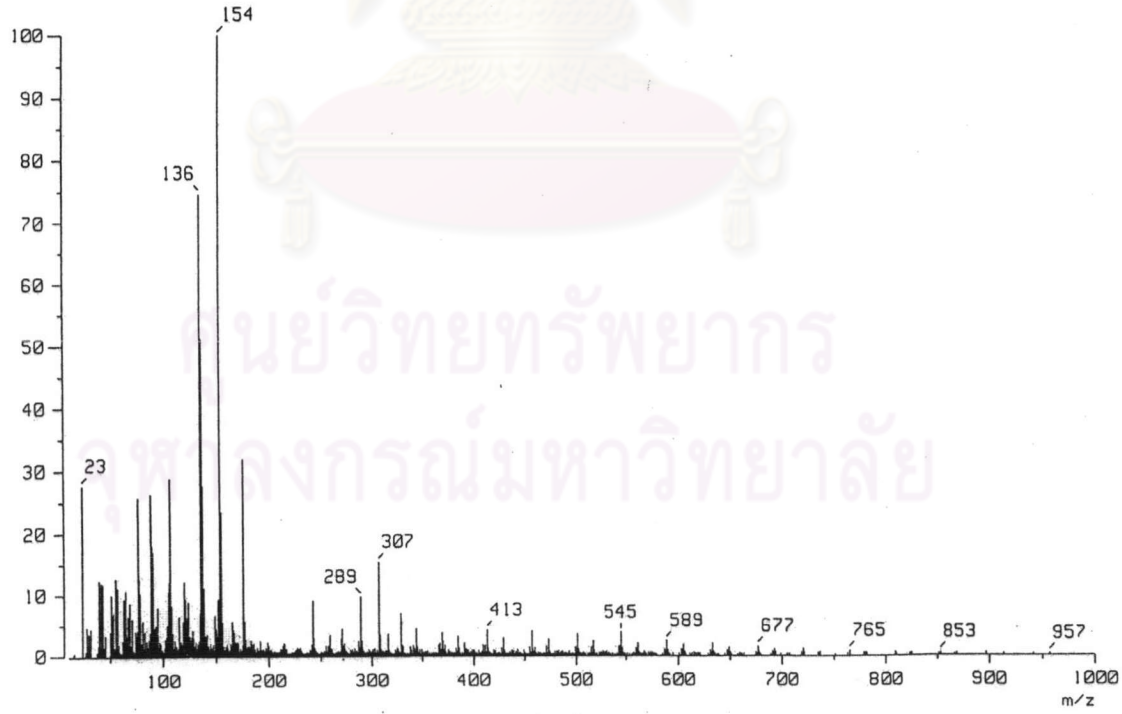


Figure 3.21 FABMS spectrum of compound **2**

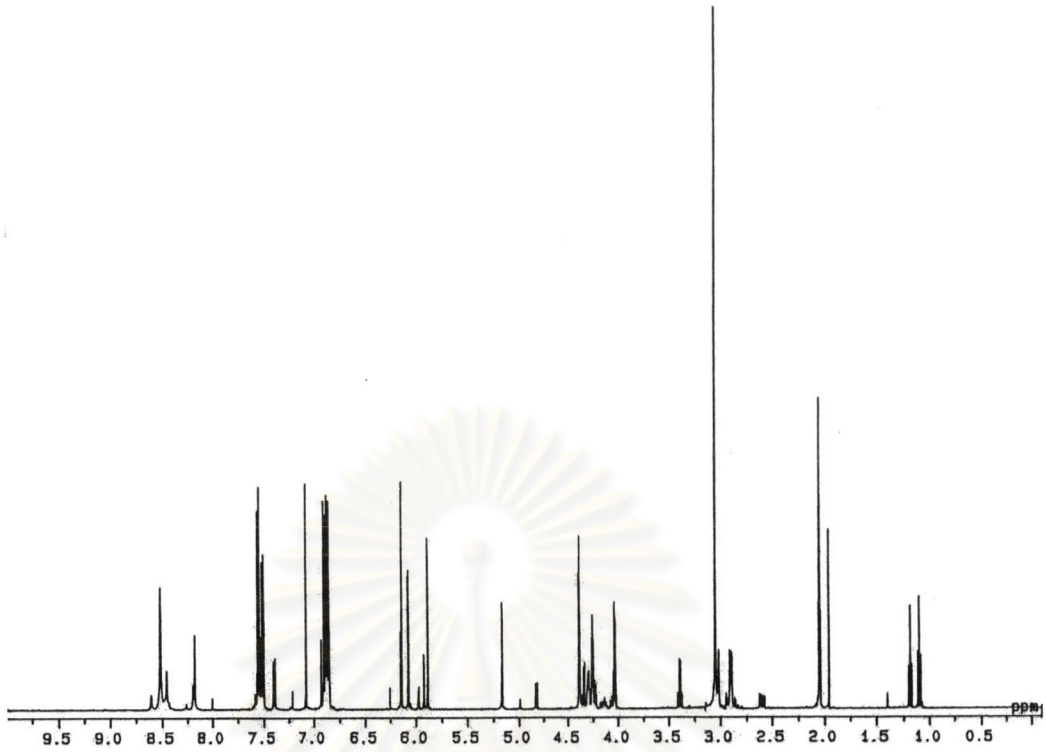


Figure 3.22 ^{1}H NMR spectrum of compound **2**

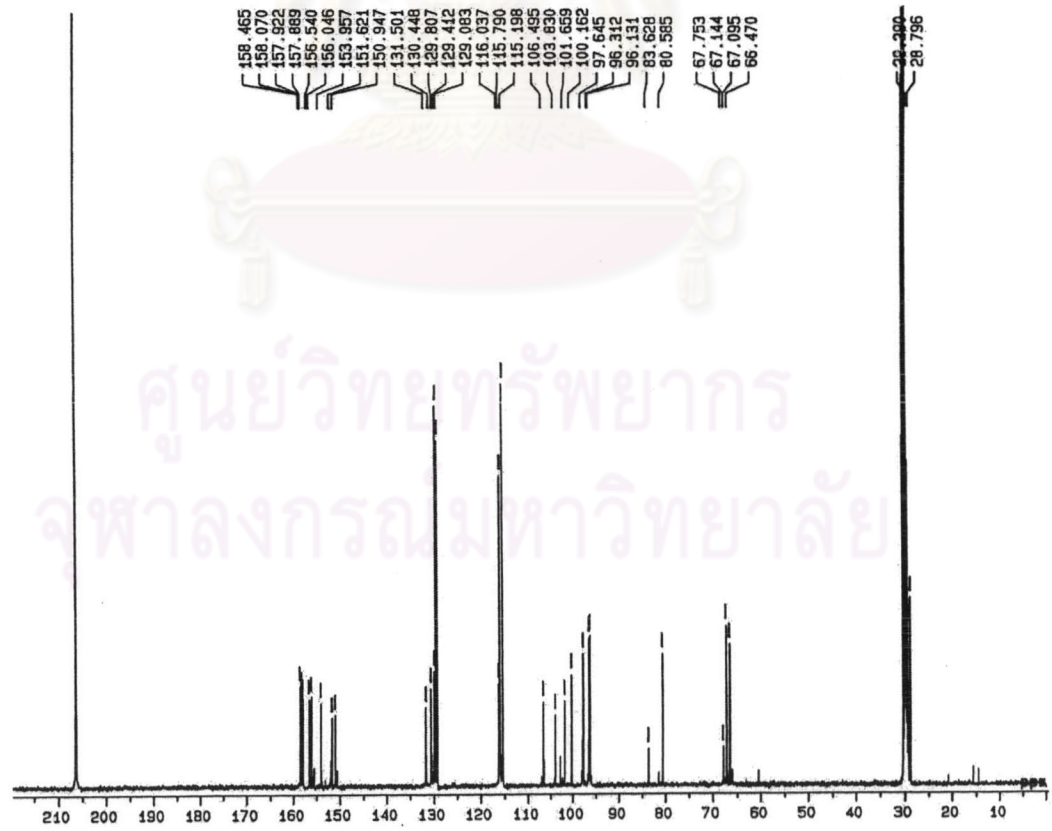


Figure 3.23 ^{13}C NMR spectrum of compound **2**

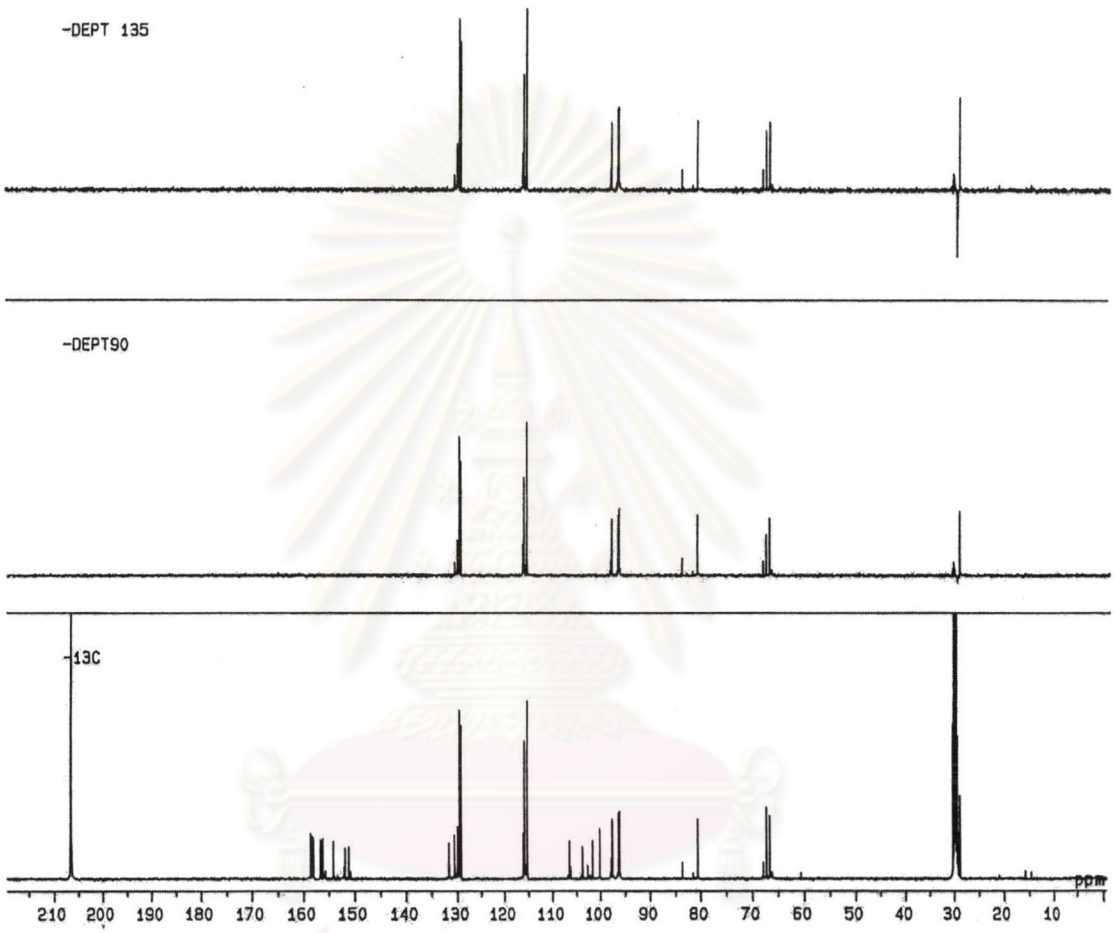


Figure 3.24 DEPT 90, 135 spectrum of compound **2**

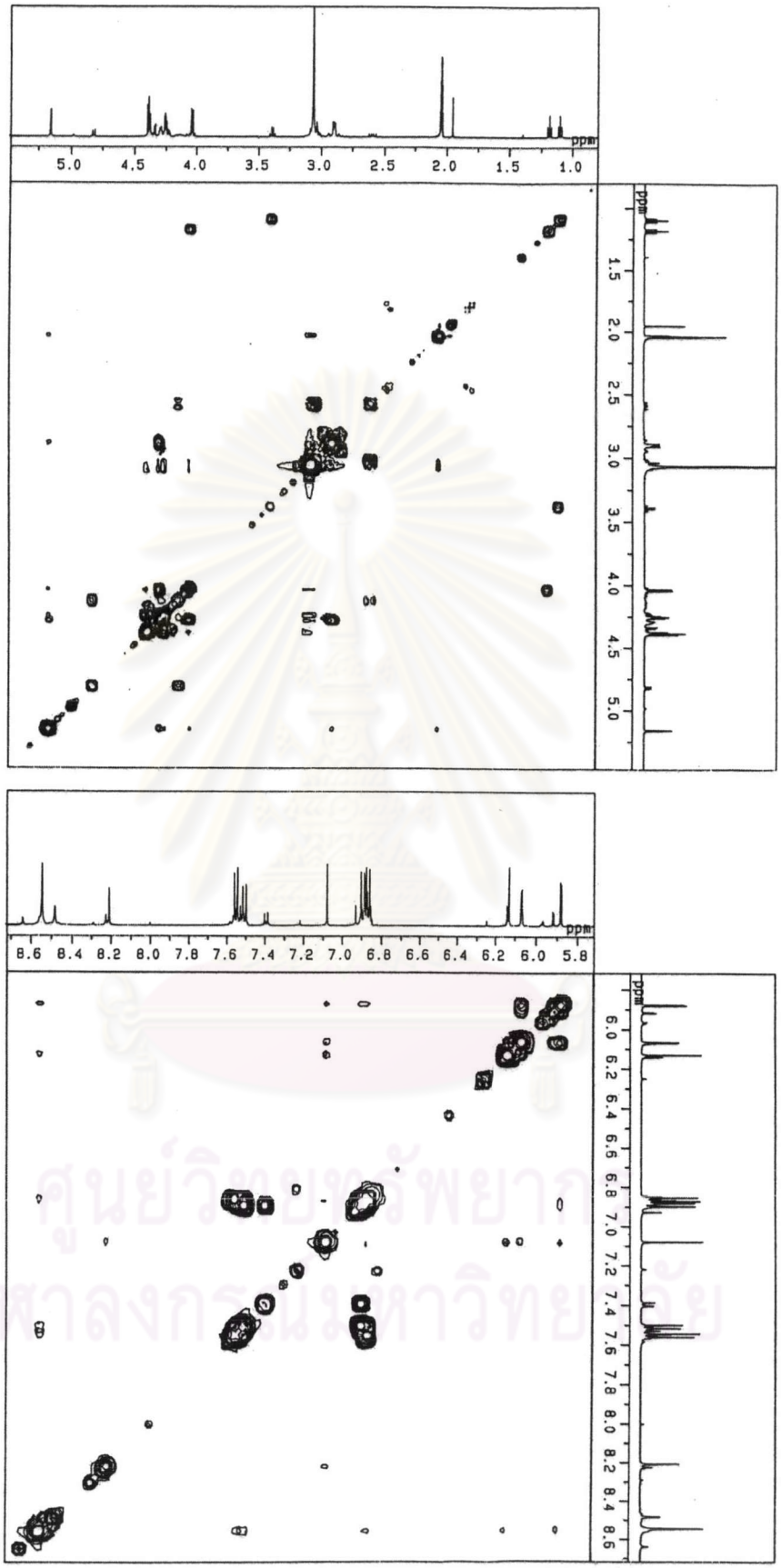


Figure 3.25 COSY spectra of compound **2**

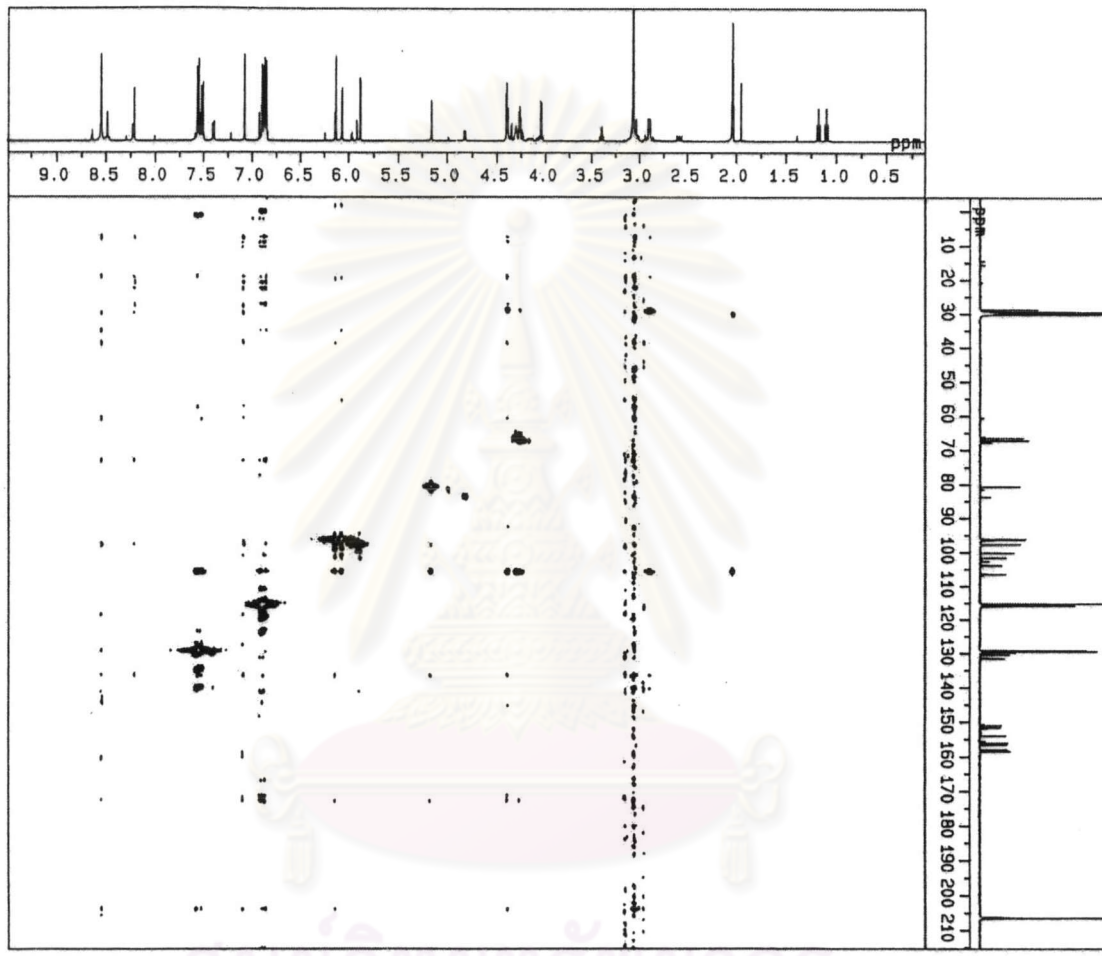


Figure 3.26 HMQC spectrum of compound **2**

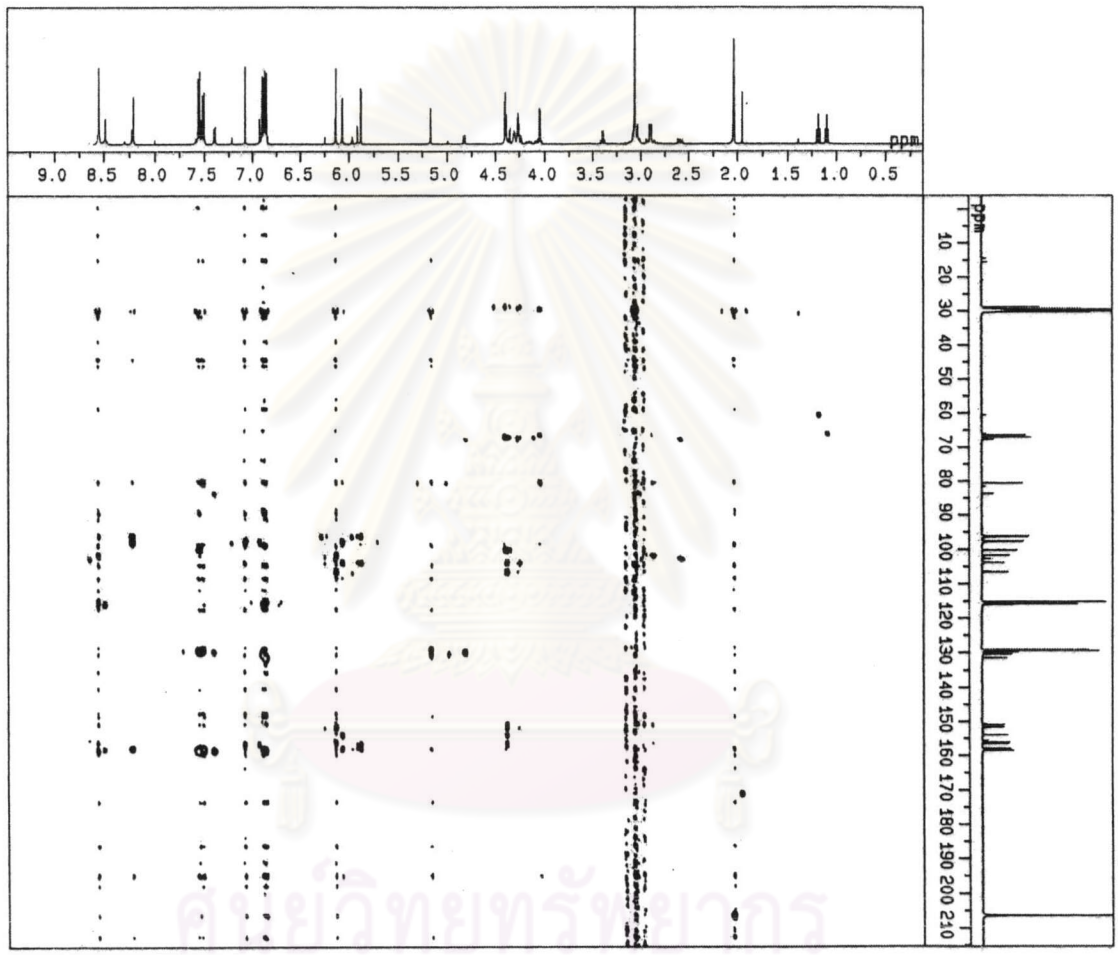


Figure 3.27 HMBC spectrum of compound **2**

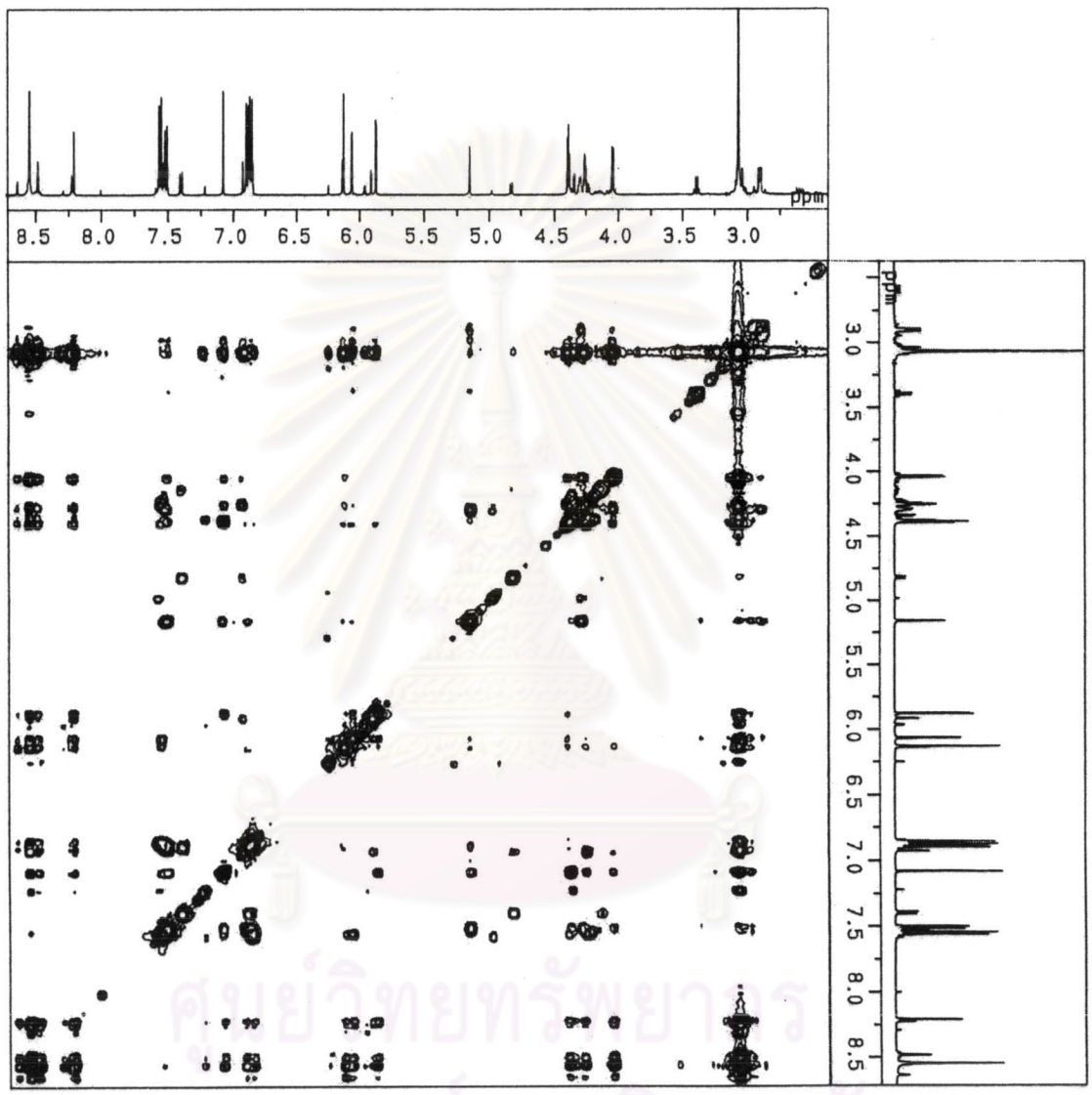


Figure 3.28 NOESY spectrum of compound **2**

3.1.6 Compound 3

Compound **3** was obtained in the same fraction of compound **2** (EtOAc/ CH_2Cl_2 4:9 to 4:1) from ethyl acetate crude extract. After recolumn chromatography by using a gradient of MeOH/$CHCl_3$ (1:9 to 4:6) and removal some of solvent, the off-white solid was observed in some fraction. This solid was washed with diethyl ether several times to afforded 18 mg of compound **3**. It had an R_f value of 0.59 (SiO_2, MeOH/ $CHCl_3$ 3:5) and decompose at 268 °C

The IR spectrum **(Figure 3.32)** of compound **3** was similar to compound **2**. It showed the strong absorption band of polyhydroxyl group at ν_{max} 3380 $cm^{-1}$and aromatic moiety was at ν_{max} 1624, 1516 and 1486 cm^{-1}.

The FABMS of compound **3 (Figure 3.33)** gave a $[M+H]^+$ peak at m/z 815, which was consistent with a trimeric proanthocyanidin. The molecular formula was assumed to be $C_{45}H_{34}O_{15}$, displaying 29 degrees of unsaturation.

The NMR spectra of compound **3** were similar to compound **2**, indicative of the presence of proanthocyanin units. The ^1H NMR spectrum **(Figure 3.34-3.35)** was operated by use acetone-d_6 as a solvent, and also showed the hydroxyl peak as well as the compound **2**. Two pair of AB system [one at δ 4.36 (1H, d, J = 3.36 Hz) and 4.39 (1H, dd, J = 3.66, 3.36 Hz), and the other at δ 4.26 (1H, dd, J = 3.36 Hz) and 4.42 (1H, d, J = 3.66 Hz), in ^1H resonance] and two ketal signals [at δ 100.3 and 101.2 in ^{13}C resonance] were observed. These evidences suggested that compound **3** was a trimeric proanthocyanidin possessing two doubly A-type linked structures. Furthermore, the ^1H NMR spectrum indicated the presence of four *meta*- coupled doublets at δ 5.89 (1H, d, J = 2.14 Hz), 5.92 (1H, d, J = 2.13 Hz), 6.04 (1H, d, J = 2.13 Hz) and 6.11 (1H, d, J = 2.44 Hz), which indicated the presence of two units of tetra substituted benzene ring. The six A_2B_2 – type doublets were observed at δ 6.88 (2H, d, J = 8.85 Hz), 6.89 (2H, d, J = 8.55 Hz), 6.94 (2H, d, J = 8.55 Hz), 7.39 (2H, d, J = 8.86 Hz), 7.58 (2H, d, J = 8.85 Hz) and 7.73 (2H, d, J = 8.85 Hz), which demonstrated the presence of three *p*-substituted phenyl moieties. On the basis of spectroscopicdata, comparison with compound **2** and those of other A-type proanthocyanidins it was suggested that compound **3** contained three flavan units, which connected by A-type linkage. The other protons were observed at δ 4.27 (1H, d, J = 5.50 Hz), 4.45 (1H, d, J = 4.58 Hz), 4.55 (1H, d, J = 4.88 Hz), 6.92 (1H,s), 6.95 (1H,s), 8.17 (1H,s), 8.21 (1H,s), 8.51 (1H,s), 8.53 (1H,s) and 8.63 (1H,s) were the

hydroxyl protons (confirm by D_2O exchange experiment, **Figure 3.36**) and protons at 2.58 (1H, dd, J = 8.85 Hz), 3.12 (1H, dd, J = 5.19, 5.49 Hz), 4.17 (1H, m) and 4.84 (1H, d, J = 8.24 Hz) were the protons of another dihydropyran unit.

The ^{13}C NMR spectrum **(Figure 3.37)** showed 37 signals of 45 carbon atoms, which consisted of 9 aliphatics and 28 aromatics (with 8 of double intensity). The DEPT spectrum **(Figure 3.38)** exhibited one methylene, 15 methines (seven of double intensity) and 21 quaternary carbons (one of double intensity). The main technique that used to assign the position of interflavonoid linkages was HMBC correlation **(Figure 3.29)**. The long-range coupling (HMBC) of H-4 proton with C-7', C-9' and H-4" proton with C-7', C-5' could be defined as (4→8, 2→O→7) and (4→6, 2→O→7) linkages, respectively. By a combination of ^1H - ^1H and ^1H - ^{13}C shift correlation spectroscopy (COSY, HMQC) and the hetero nuclear multiple-bond correlation spectroscopy (HMBC), a planar structure of compound **3** was established, the signals of proton and carbon could be definitively assigned and the carbon signals of this compound was also compared with related compound[37] (parameritannin A-2) in **Table 3.5** (All the spectra are shown in **Figure 3.39 – Figure 3.44**). The NMR data did not provide unambiguous information concerning the stereochemistry of compound **3**. However, the presence of coupling constant (J = 8.24 Hz) at H-2' was indicated the *trans*-relationship between H-2' and H-3'. The NOESY correlation **(Figure 3.30)** did not show the correlation of hydroxyl groups at C-3 and C-3" with the phenyl groups at C-2 and C-2", respectively. These results suggested the hydroxyl groups were in difference plane with phenyl groups. So, the possible structure of compound **3** was proposed to be afzelechin-(4→6, 2→O→7)-afzelechin-(4→8, 2→O→7)-afzelechin. **(Figure 3.31)** Computational structure searching suggested that this compound was not previously reported; consequently, it was a new compound.

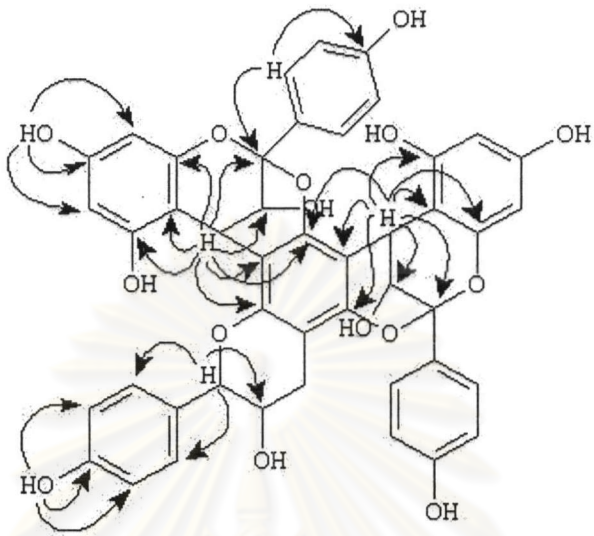


Figure 3.29 Selected HMBC correlation of compound **3**

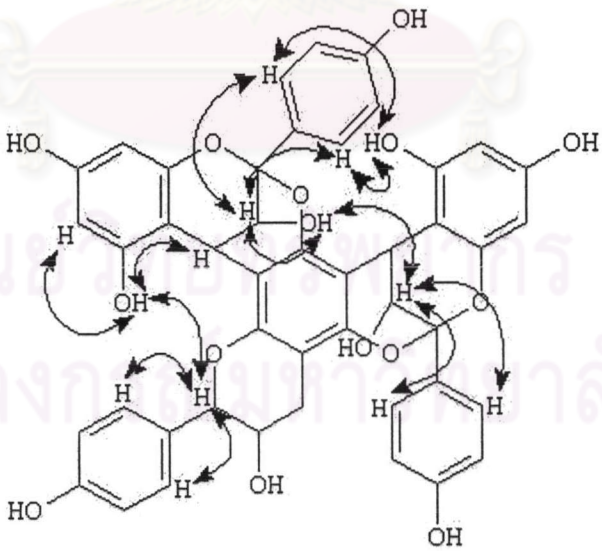
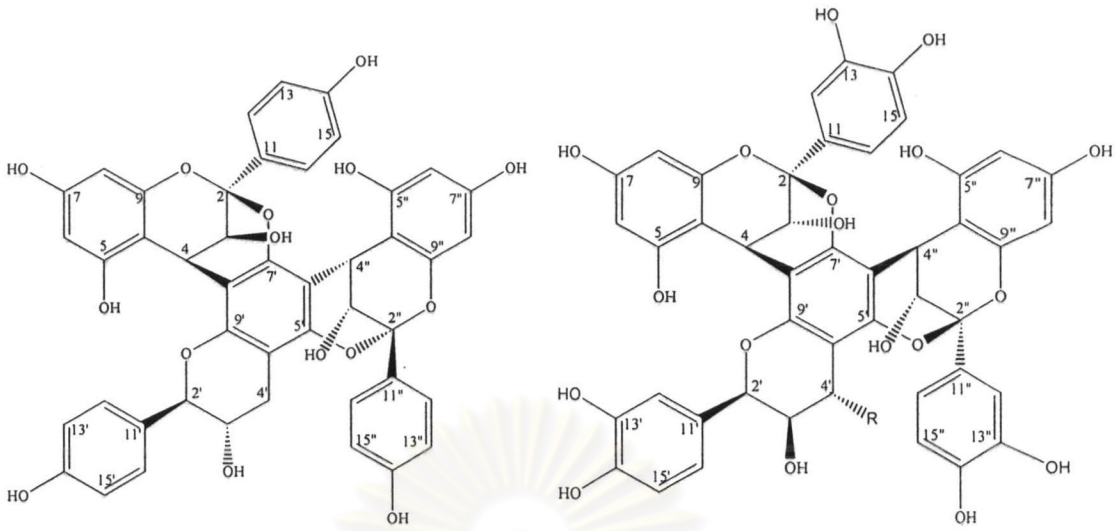


Figure 3.30 Selected NOESY correlation of compound **3**



(a) (b)

Figure 3.31 (a) The possible structure of compound **3**.
(b) Structure of parameritannin A-2.

Table 3.5 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **3**[a]

No.	δH (*J* in Hz)	δC [b]	^{1}H-^{13}C connectivities (HMBC)	δC [c]
2		101.2		100.8
3	4.39 (1H, dd, *J* = 3.66, 3.36)	66.7	102.9, 107.9	66.4
4	4.36 (1H, d, *J* = 3.36)	28.8	66.7, 101.2, 102.9, 107.9, 146.3, 149.0, 153.5, 156.7	28.9
5		156.7		157.8
6	5.89 (1H, d, *J* =2.14)	98.2	96.3, 102.9, 156.7, 158.1	98.7
7		158.1		157.9
8	6.11 (1H, d, *J* =2.44)	96.3	98.2, 102.9, 153.5, 158.1	98.8
9		153.5		153.7
10		102.9		104.2
11		130.4		131.2
12	7.73 (2H, d, *J* = 8.85)	129.5	101.2, 159.0	115.6
13	6.94 (2H, d, *J* = 8.55)	115.6	130.4	145.7
14		159.0		147.0
15	6.94 (2H, d, *J* = 8.55)	115.6	130.4	116.1

Table 3.5 (cont.) ^{1}H NMR (500 MHz) and ^{13}C nmr (125 MHz) data of compound **3**[a]

No.	δH (*J* in Hz)	δC [b]	^{1}H-^{13}C connectivities (HMBC)	δC [c]
16	7.73 (2H, d, *J* = 8.85)	129.5	101.2, 159.0	119.8
2'	4.84 (1H, d, *J* = 8.24)	83.6	67.6, 129.8	78.8
3'	4.17 (1H, m)	67.6		72.1
4'a 4'b	2.58 (1H, dd, *J* = 8.85) 3.12 (1H, dd, *J* = 5.19, 5.49)	29.3	67.6, 149.0 67.6, 83.6, 149	38.8
5'		149.5		149.7
6'		108.0		107.0
7'		146.3		145.5
8'		107.9		108.1
9'		149.0		150.3
10'		103.7		107.6
11'		129.7		131.5
12'	7.39 (2H, d, *J* = 8.86)	129.8	83.6, 158.5	116.7
13'	6.89 (2H, d, *J* =8.55)	116.1	129.7	145.8
14'		158.5		146.3
15'	6.89 (2H, d, *J* =8.55)	116.1	129.7	116.1
16'	7.39 (2H, d, *J* = 8.86)	129.8	83.6, 158.5	121.3
2"		100.3		100.2
3"	4.26 (1H, dd, *J* = 3.36)	67.0	103.1	68.0
4"	4.42 (1H, d, *J* = 3.66)	29.1	67.0, 100.3, 103.1, 108.0, 146.3, 149.5, 153.8, 156.6	29.5
5"		156.6		156.2
6"	5.92 (1H, d, *J* = 2.13)	98.2	96.3, 103.1, 156.6, 158.1	98.4
7"		158.1		157.9
8"	6.04 (1H, d, *J* = 2.13)	96.3	98.2, 103.1, 153.8, 158.1	96.6
9"		153.8		154.1
10"		103.1		103.9
11"		131.2		131.9
12"	7.58 (2H, d, *J* = 8.85)	129.5	100.3, 158.6	116.5

Table 3.5 (cont.) ^{1}H NMR (500 MHz) and ^{13}C nmr (125 MHz) data of compound **3**[a]

No.	δH (*J* in Hz)	δC [b]	^{1}H-^{13}C connectivities (HMBC)	δC [c]
13"	6.88 (2H, d, *J* = 8.85)	115.3	131.2	144.7
14"		158.6		146.2
13"	6.88 (2H, d, *J* = 8.85)	115.3	131.2	116.0
16"	7.58 (2H, d, *J* = 8.85)	129.5	100.3, 158.6	119.6
OH-3	4.55 (1H, d, *J* = 4.88)		101.2	
OH-5	6.95 (1H, s)		98.2, 102.9	
OH-7	8.21 (1H, s)		96.3, 98.2, 158.1	
OH-14	8.63 (1H, s)		115.6, 159.0	
OH-3'	4.27 (1H, d, *J* = 5.50)		67.6, 83.6	
OH-14'	8.51 (1H, s)		116.1, 158.5	
OH-3"	4.45 (1H, d, *J* = 4.58)		100.3	
OH-5"	6.92 (1H, s)		98.2, 103.1, 156.6	
OH-7"	8.17 (1H, s)		96.3, 98.2, 158.1	
OH-14"	8.53 (1H, s)		115.3, 158.6	

[a] Measured in Me_2CO-d_6 , [b] Carbon signals of compound **3** , [c] Carbon signal of parameritannin A-2.

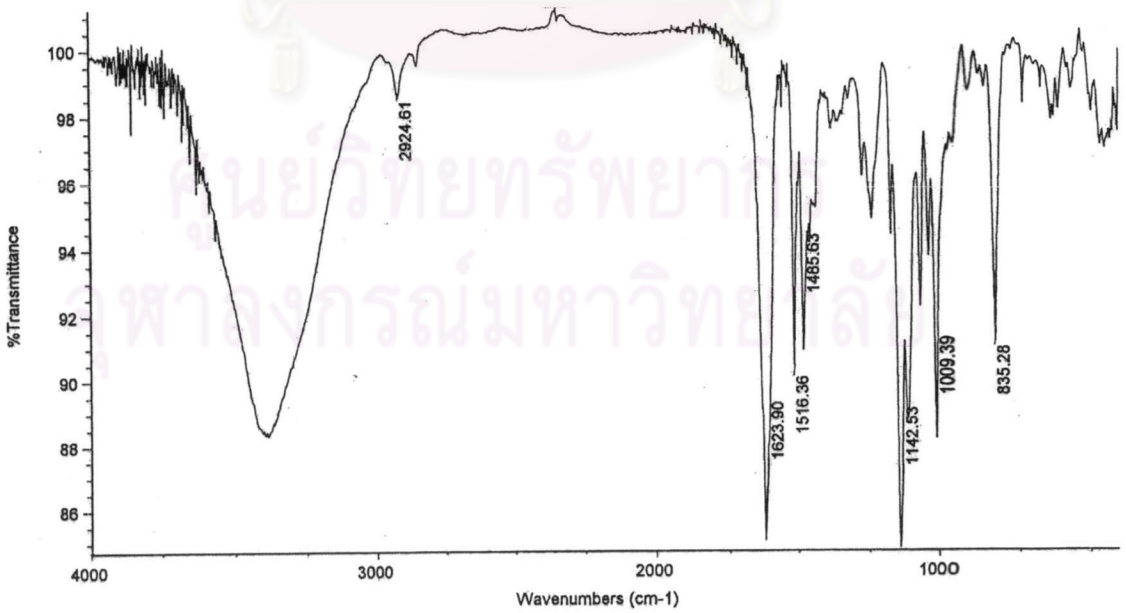


Figure 3.32 IR spectrum of compound **3**

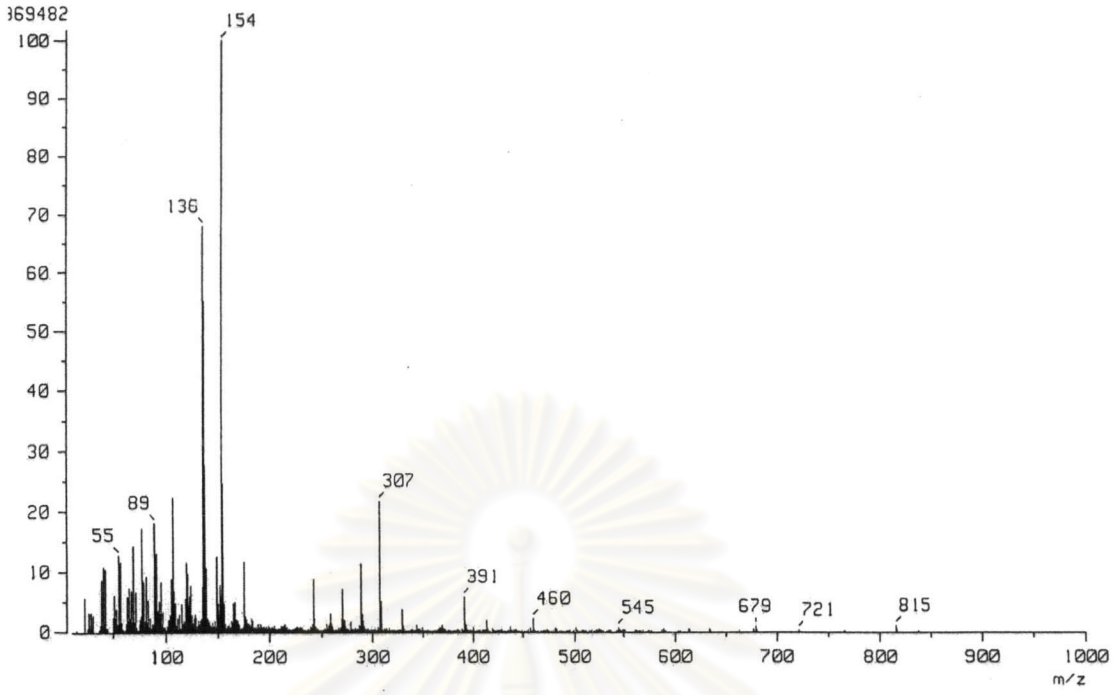


Figure 3.33 FABMS spectrum of compound **3**

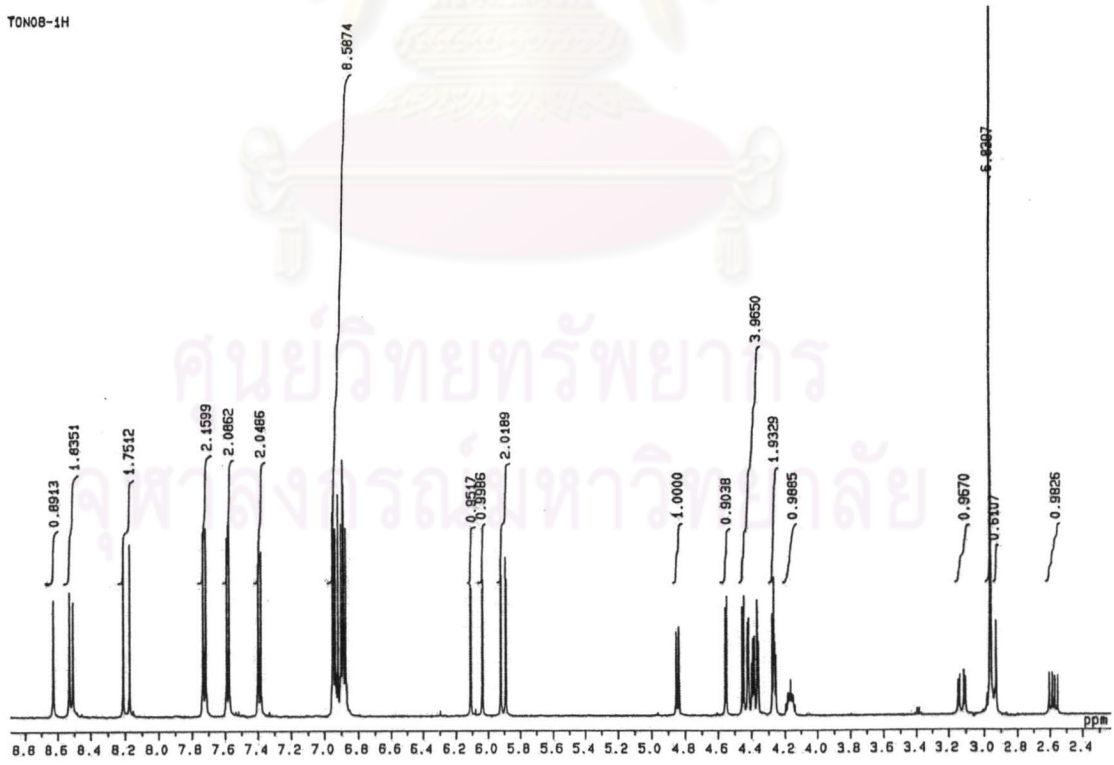


Figure 3.34 ^{1}H NMR spectrum of compound **3**

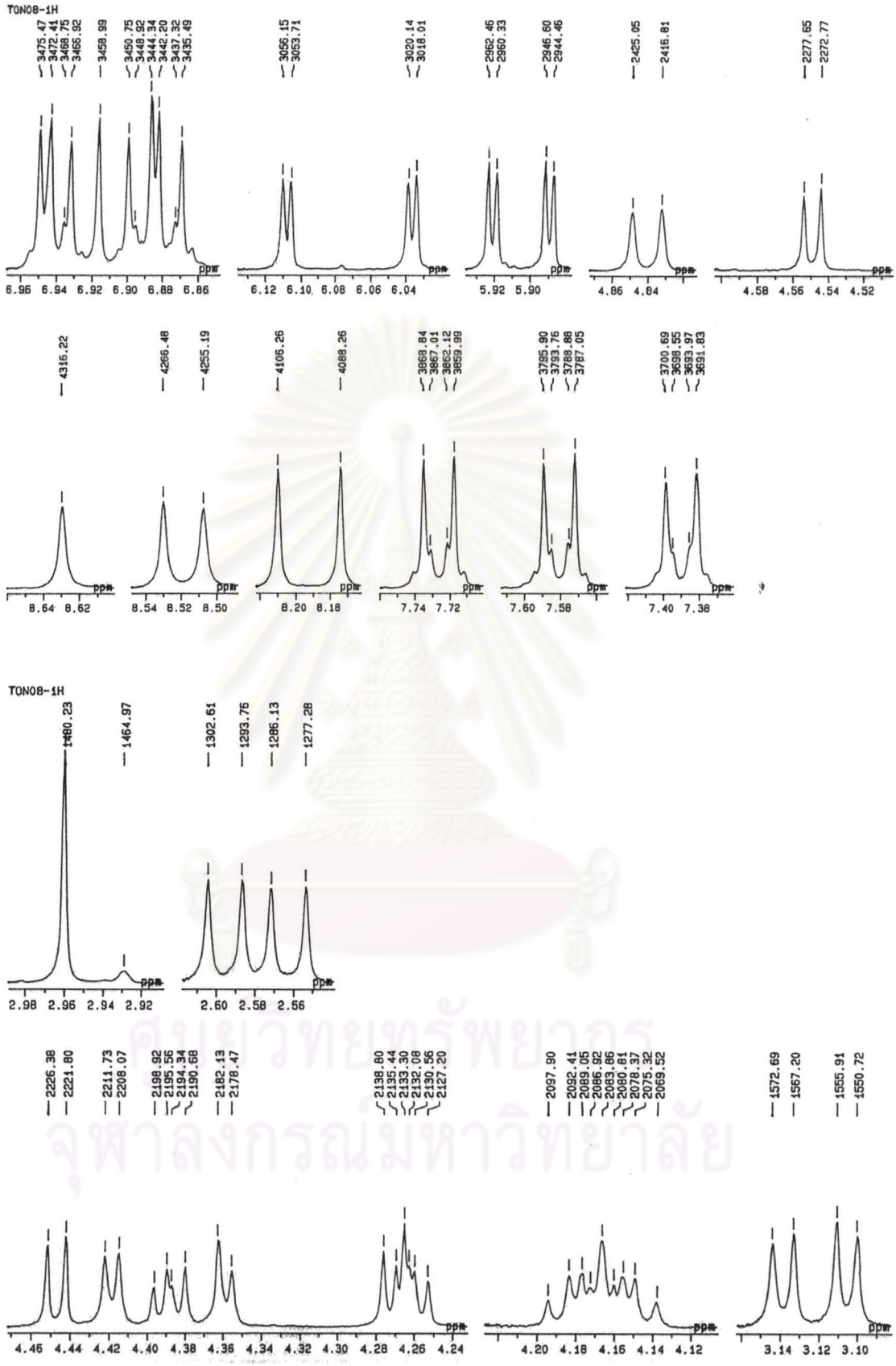


Figure 3.35 ^{1}H NMR spectrum of compound **3** (expansion)

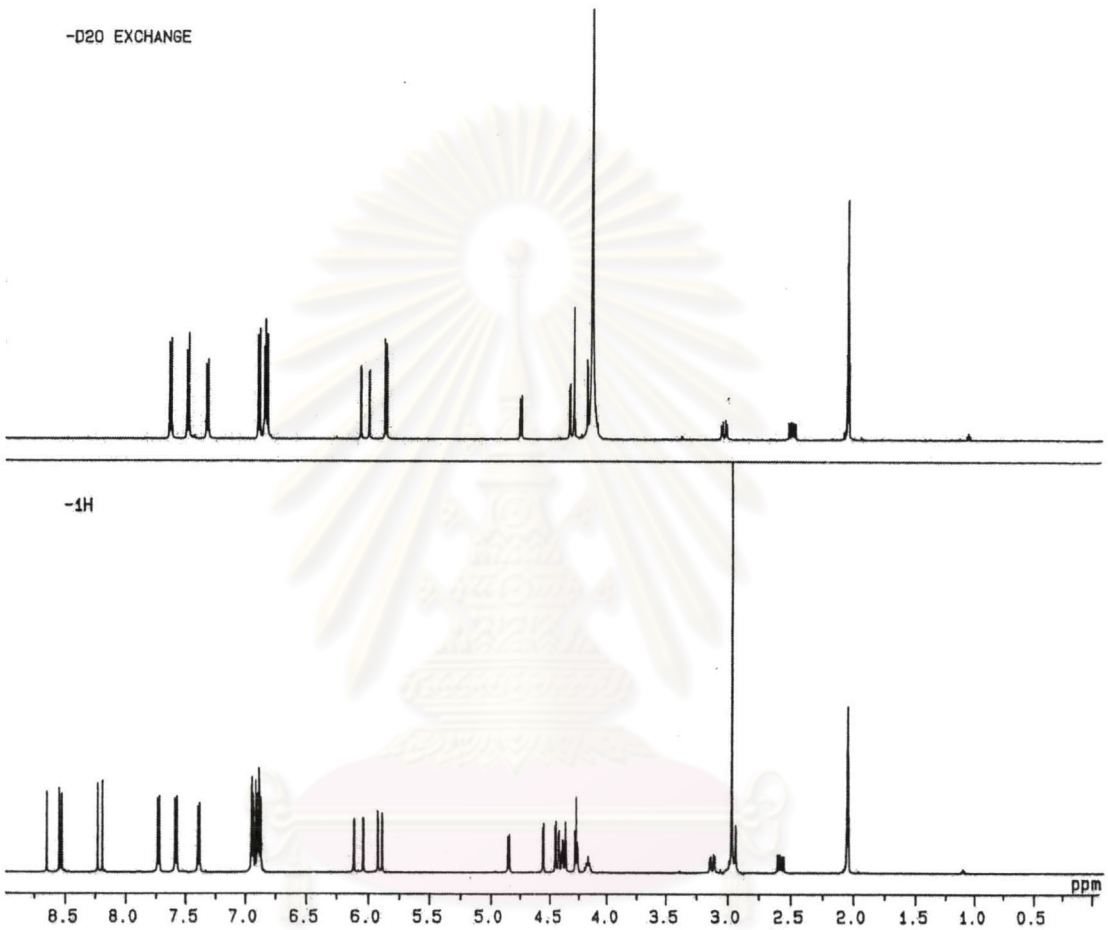


Figure 3.36 D_2O exchange spectrum of compound **3**

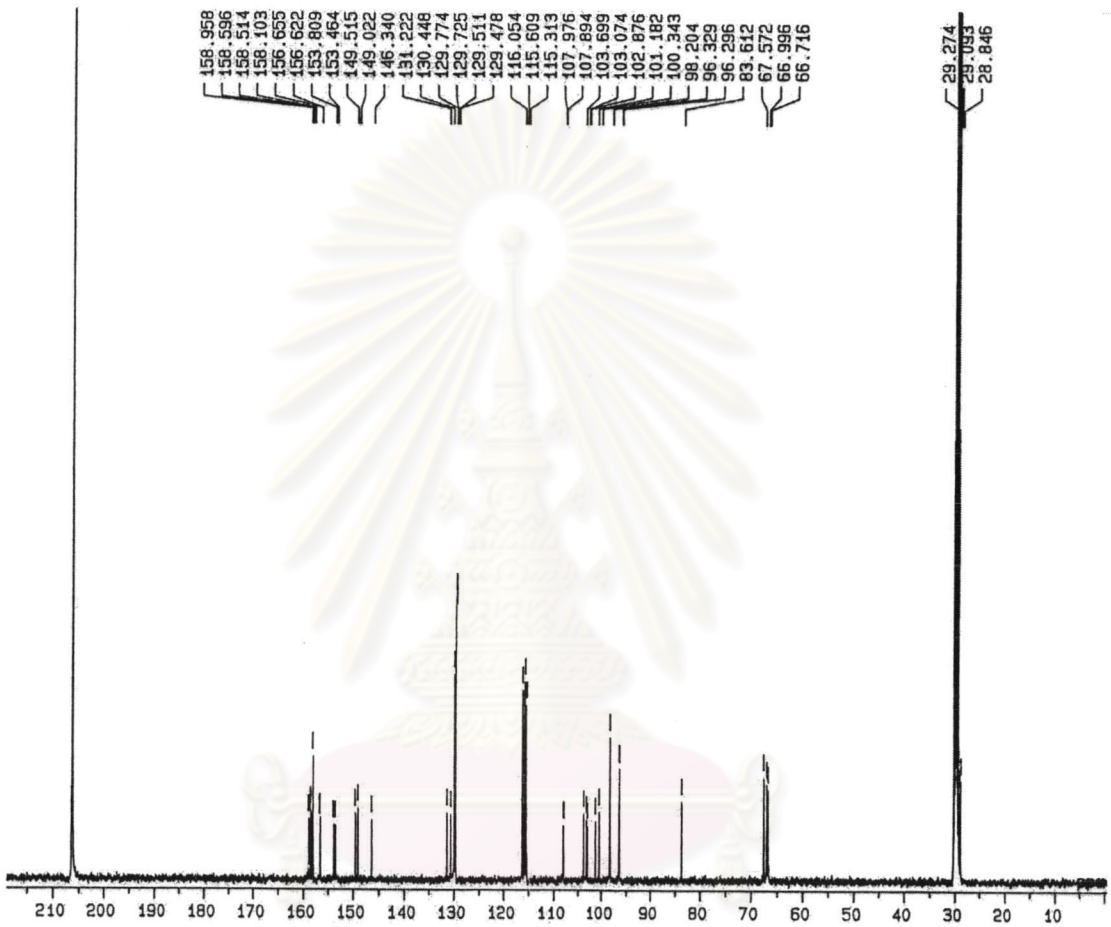


Figure 3.37 ^{13}C NMR spectrum of compound 3

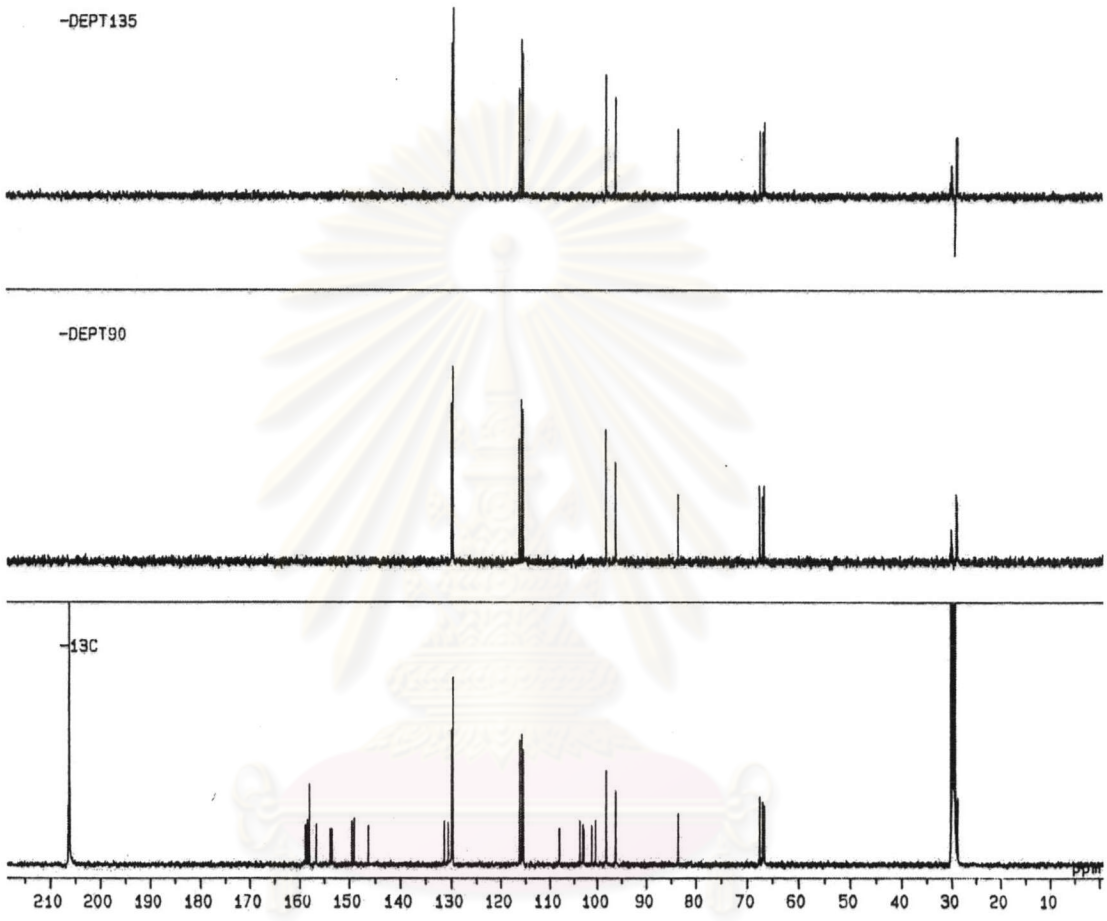


Figure 3.38 DEPT 90, 135 spectrum of compound **3**

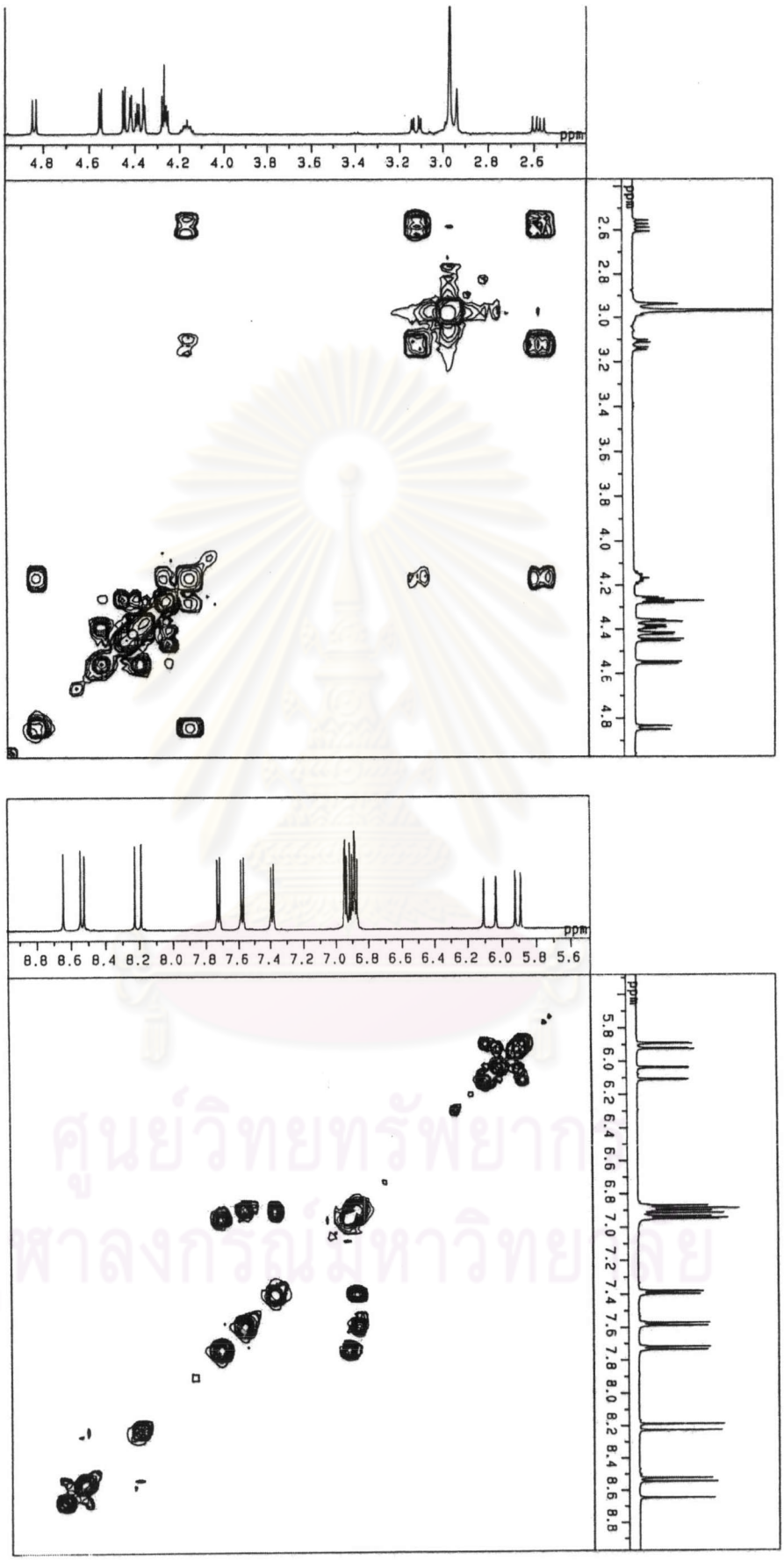


Figure 3.39 COSY spectra of compound **3**

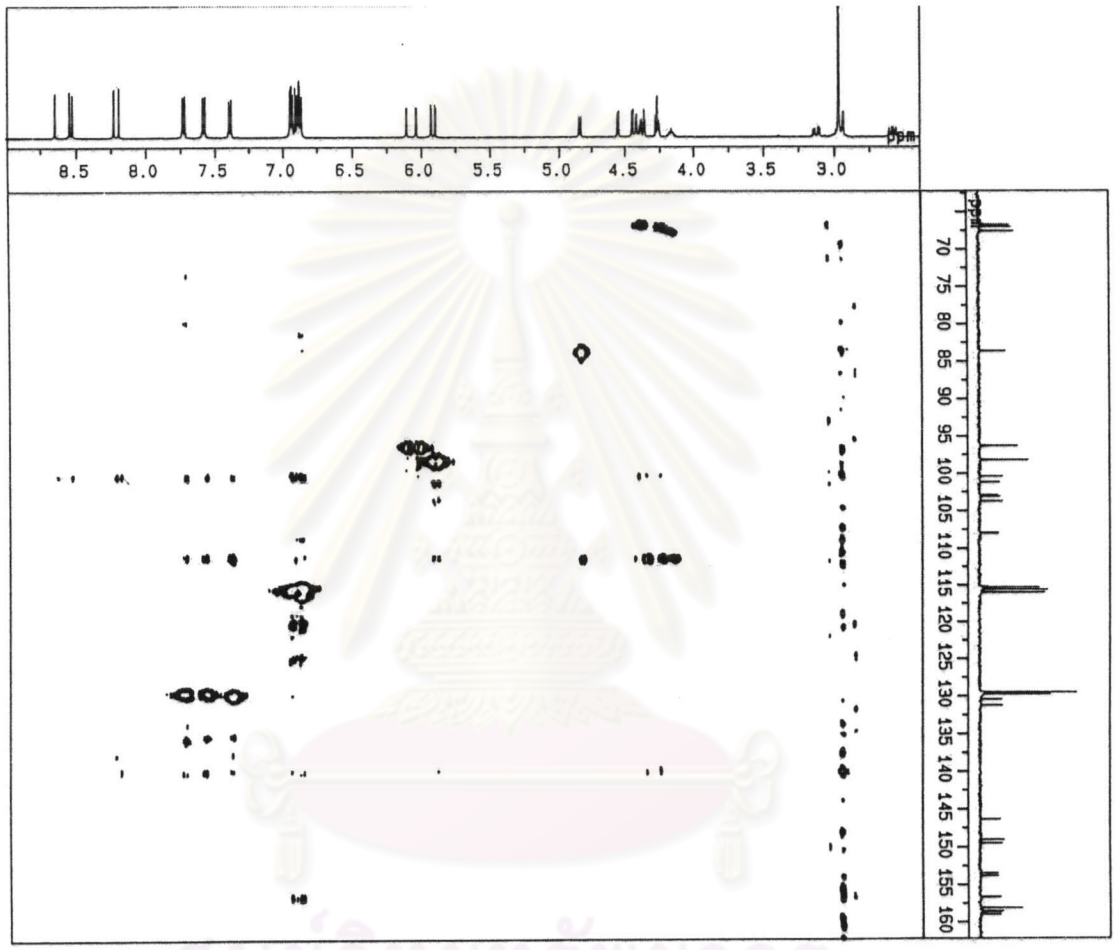


Figure 3.40 HMQC spectrum of compound **3**

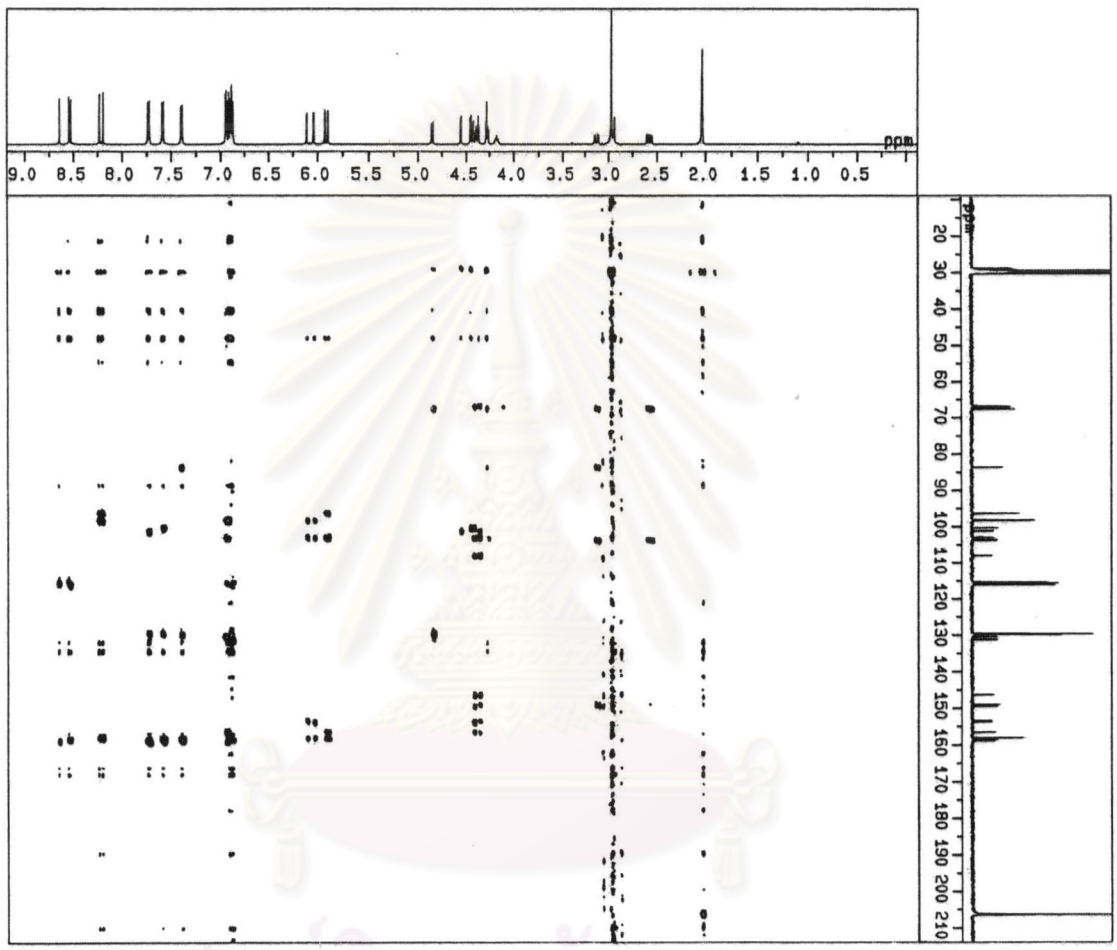


Figure 3.41 HMBC spectrum of compound **3**

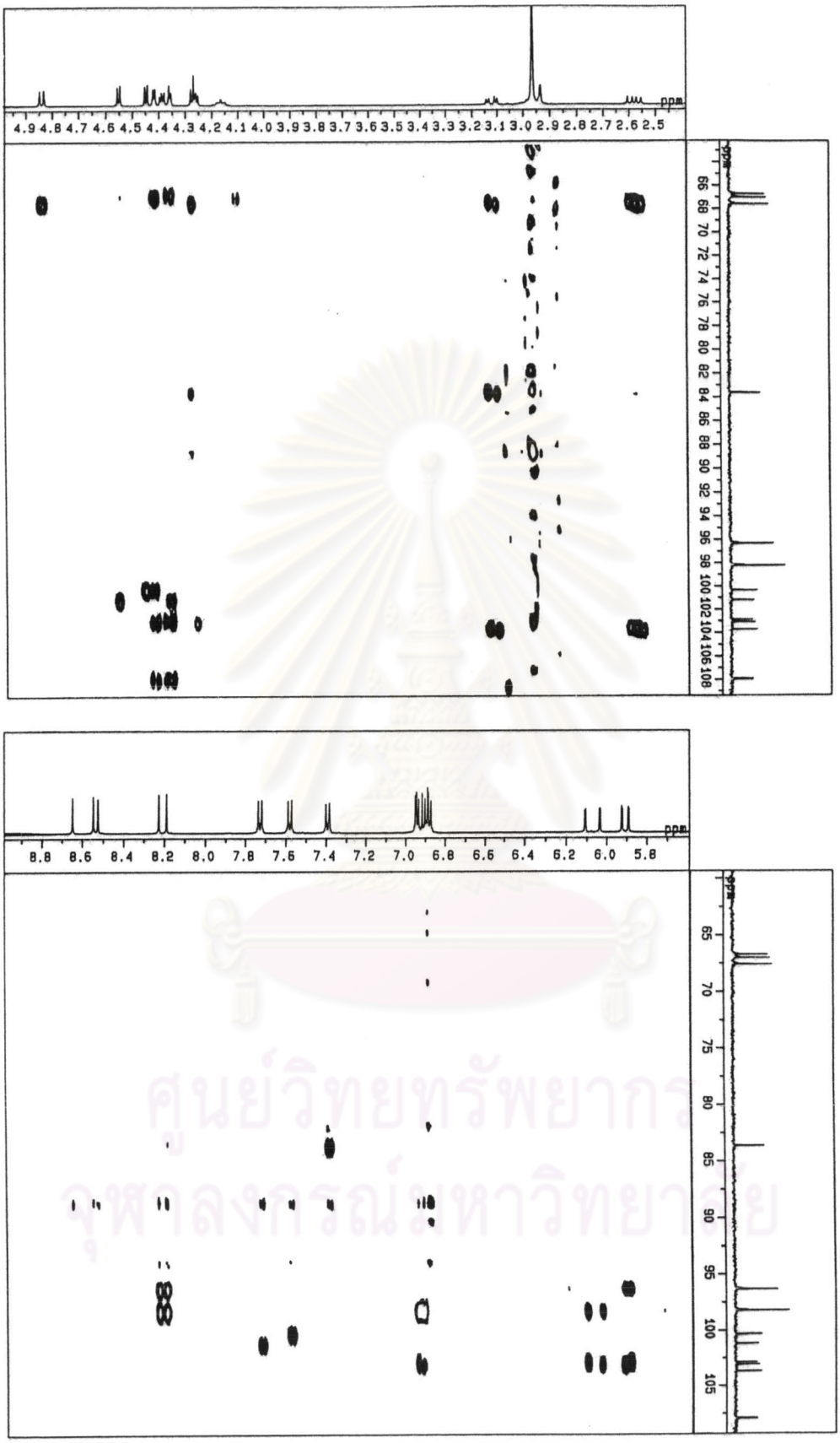


Figure 3.42 HMBC spectrum of compound **3** (expansion)

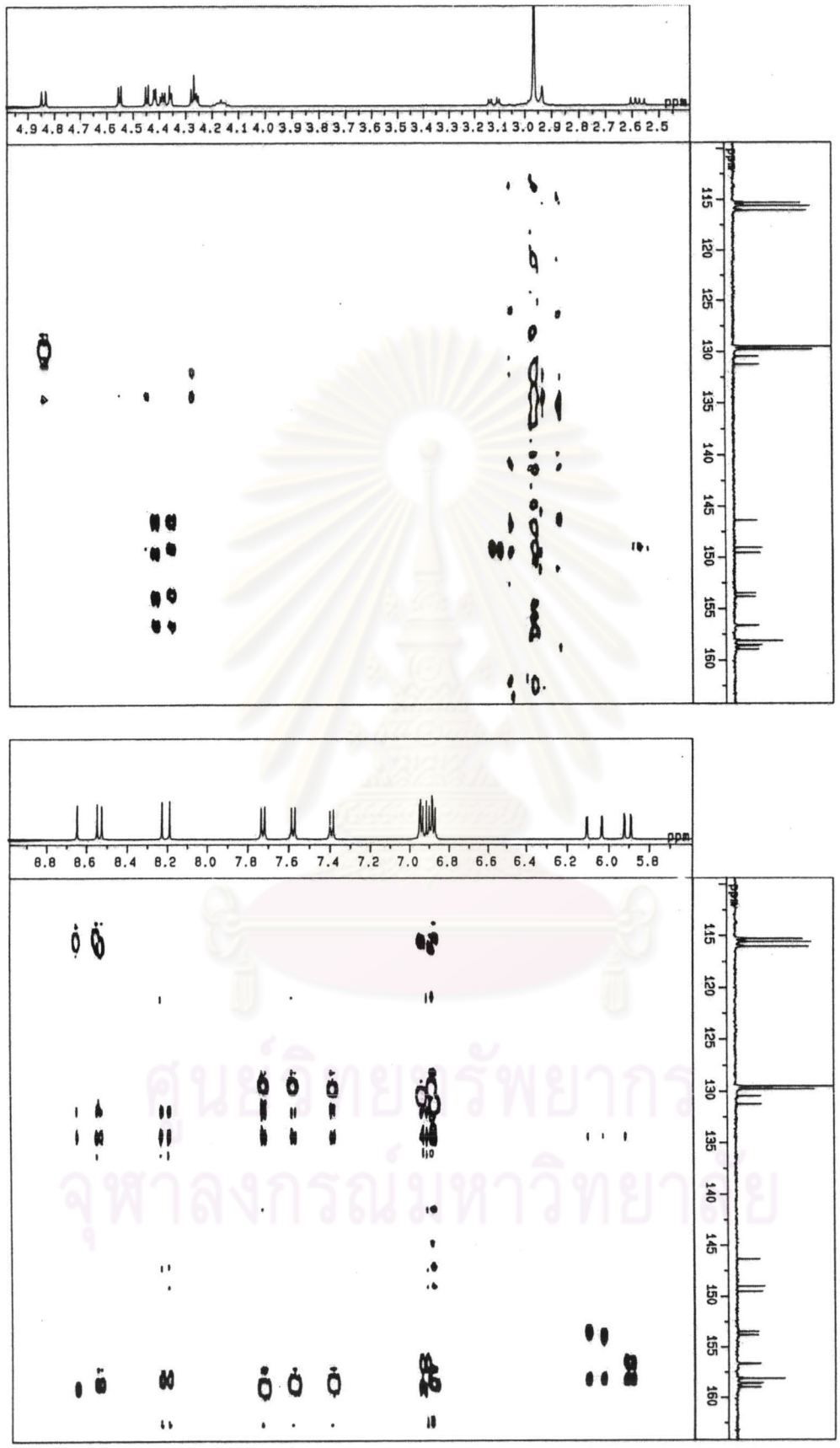


Figure 3.43 HMBC spectrum of compound **3** (expansion)

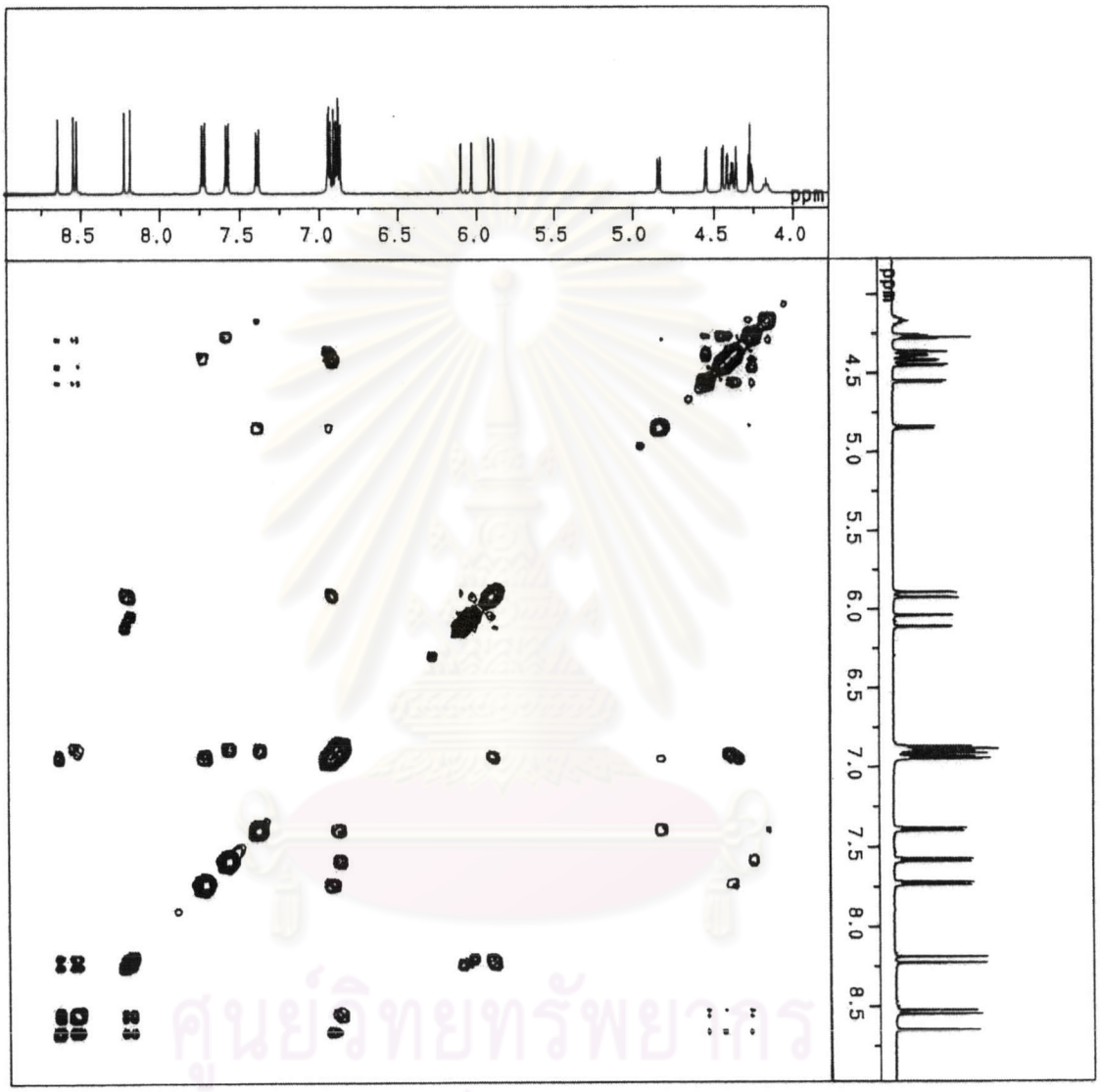


Figure 3.44 NOESY spectrum of compound **3**

3.2 The Results of Biological Activities of Isolated Compounds and Mixture

According to the preliminary test with DPPH (TLC autographic assay), mixtures **3**, compound **2** and compound **3** showed the positive results. There were chosen for further performed by spectrophotometric assay, which the results were displayed in **Table 3.6**.

Table 3.6 Free Radicals Scavenging Activity of Isolated Compounds and Mixture on DPPH

Isolated Compounds	Conc. (ppm)	% radical scavenging
Mixture **3**	62.5	26.49
	125	47.29
	250	69.90
	500	89.10
Compound **1**	125	10.01
	250	14.87
	500	17.57
Compound **2**	15.63	16.58
	31.25	30.59
	62.5	37.55
	125	57.97
	250	70.85
	500	79.25
Compound **3**	15.63	16.05
	31.25	31.10
	62.5	44.37
	125	62.60
	250	70.97
	500	81.47
Vitamin E	31.25	14.16
	62.5	40.42
	125	78.73
	250	94.16

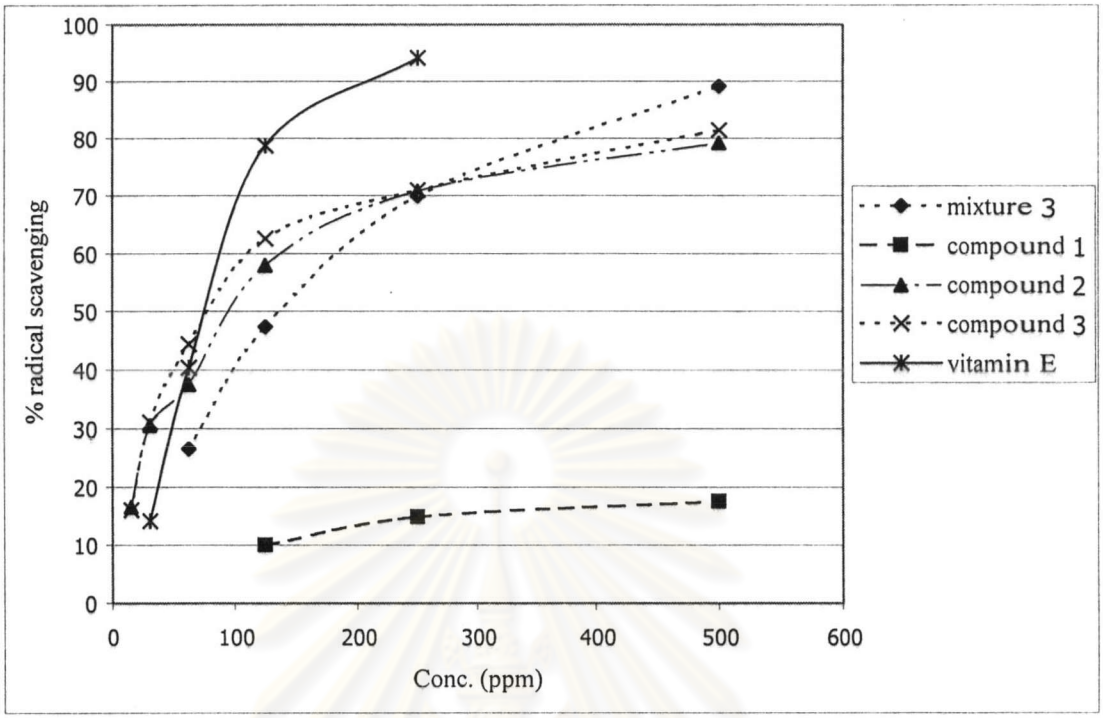


Figure 3.45 Scavenging activity of isolated compounds, mixture and vitamin E

Table 3.7 IC_{50} on Free Radicals Scavenging Activity of Isolated Compounds and Mixture on DPPH

Isolated Compound	IC_{50} (ppm)
Mixture **3**	134.96
Compound **1**	> 500
Compound **2**	98.86
Compound **3**	83.49
Vitamin E	74.49

Compound **2** and compound **3** showed the attractive results on DPPH assay. Both IC_{50} are in the vicinity with vitamin E, so they were selected for further investigation with Superoxide dismutase , Xanthine Oxidase Inhibition and Ferric thiocyanate assay. The results of these assays were exhibited in **Table 3.8**, **Table 3.9** and **Figure 3.46**, respectively.

Table 3.8 Superoxide dismutase (SOD) activity of Isolated Compounds

Isolated compounds	Conc. (ppm)	% SOD
Compound **2**	10	77.90
	50	80.37
	100	81.12
	250	82.83
Compound **3**	10	80.05
	50	81.87
	100	82.38
	250	84.12
BHA	10	80.52
	50	83.86
	100	84.06
	250	84.53

Table 3.9 Xanthine Oxidase Inhibition activity of Isolated Compounds

Isolated compounds	Conc. (ppm)	% Inhibition
Compound **2**	10	70.47
	50	74.83
	100	75.10
	250	77.64
Compound **3**	10	53.24
	50	70.24
	100	72.16
	250	74.00
Allopurinol (Standard)	10	68.19
	50	74.62
	100	75.47
	250	80.72

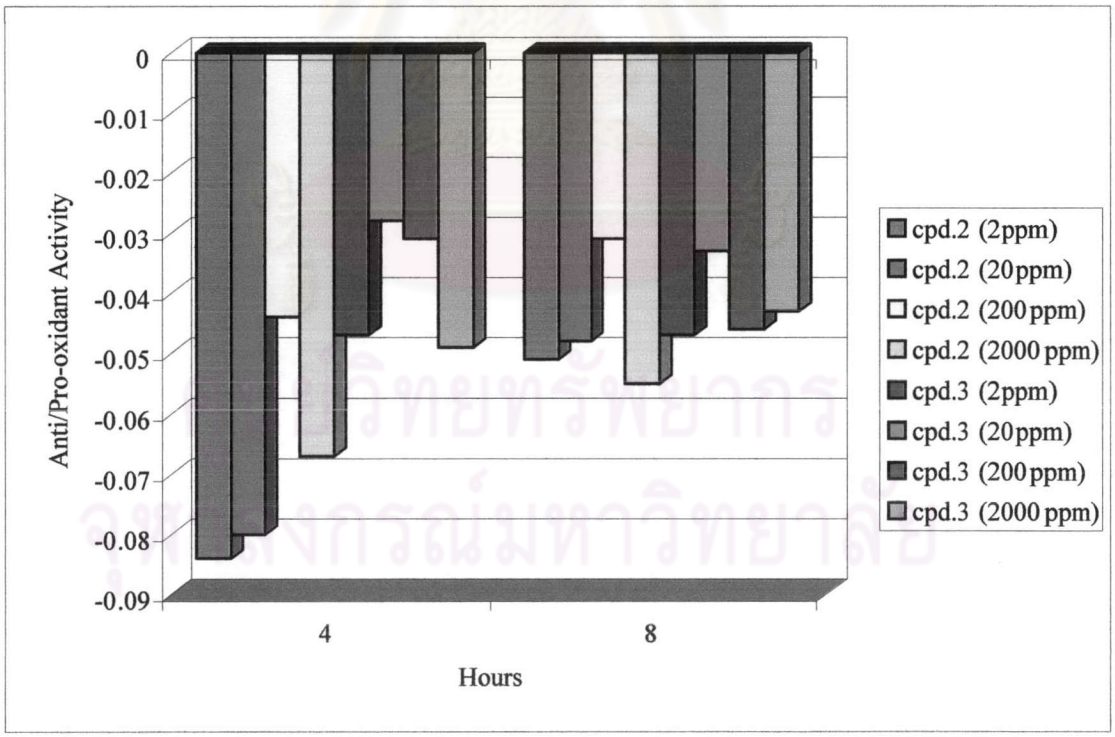


Figure 3.46 Activity of isolated compound on Ferric thiocyanate assay

In addition, mixture **3**, compound **2** and compound **3** were tested for inhibitory effect on KB cell lines but they showed noncytotoxic to this tumour cell. The result was displayed in **Table 3.10**

Table 3.10 Cytotoxic Activity Against KB Cell lines by MTT Assay

Sample	IC_{50} (ppm)
Mixture **3**	> 100
Compound **2**	> 100
Compound **3**	> 100

In terms of biological activities:

1. On DPPH, mixture **3,** compound **2** and compound **3** showed significant activity with IC_{50} 134.96, 98.86 and 83.49 μg/ml, respectively

2. Ferric thiocyanate assay, compound **2** and **3** showed the antioxidant activity, they seemed to inhibit the radical rather than enhance the initiated of radical.

3. Superoxide dismutase and Xanthine oxidase inhibition:

Compound **2** and **3** showed strong activity with SOD assay and also showed the inhibition effect on xanthine oxidase that suggested they may inhibit xanthine oxidase before generated $O_2^{\bullet-}$ in SOD assay. However, the cause of decreasing of $O_2^{\bullet-}$ may occur as co-activities, inhibition enzyme and scavenging activity on $O_2^{\bullet-}$.

4. Inhibition effect for Tumer Cell Lines.

Mixture **3,** compound **2** and **3** did not showed the inhibition for tumour cell. Their IC_{50} values are greater than 100 ppm (positive result must be $\geq$ 4 ppm) that may suggested that they have low toxicity to the cell.

From biological activities data, compound **2** and **3** (new compound) showed high % xanthine oxidase inhibition (70.49, 53.24/ 10 ppm) among the isolated compounds from this plant and closed to allopurinol (68.19/ 10 ppm) which used as oral drug for treating gout disease. These two compounds seemed to be nontoxic to cell so they might be developed to be drug for treating gout in the future.