CHAPTER 3

Results and Discussion

3.1 Structural elucidation of pure compounds in *Vernonia scandens* aerial parts

In a continuation study of the phytochemical constituents from CH_2Cl_2 extract of *V. scandens* aerial parts afforded six known compounds, lupeol palmitate (**134**), lupeol acetate (**135**), eugenol (**98**), macelignan (**136**), and mixture of β-sitosterol (**137**) and stigmasterol (**70**). The biological activities of isolated compounds in this plant which was known compounds in general. Therefore, the bioactivities investigations of these compounds were not test in this research. Biological activities of compounds were reported from the literature as shown in Table 3.1.

Table 3.1 Biological activities of compounds from the aerial parts of *V. scandens*

Compounds	Activities [References]
Lupeol palmitate (**134**)	Antiinflammatory constituents in the croton oil-induced ear oedema test [35]
Lupeol acetate (**135**)	Cytotoxic [36, 37] - HuCCA-1 cell line with ED_{50} > 100 μg/ml [36] - KB cell line with ED_{50} > 100 μg/ml [36] - MCF-7 cell line with IC_{50} 1.04 μg [37] - HCT116 cell line with IC_{50} 2.89 μg [37] *ED_{50} > 100 μg/ml denotes inactive cytotoxic activity* Anti inframmatory effect [38, 39] - Inhibited the proliferation of mitogen induced PBMCs with IC_{50} 8 g/ml [39]
Eugenol (**98**)	Inhibits human monoamine oxidase A (MAOA) with IC_{50} 10 μM [40]

Table 3.1 (Continued)

Compounds	Activities [References]
Macelignan (**136**)	Against other oral microorganisms [41] -*Streptococcus sobrinus*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Lactobacillus acidophilus* and *Lactobacillus casei* in the MIC range of 2–31.3 μg/ml Antioxidant and anti-inflammatory activities [42] Increase caspase-3 activity [43] Induced internucleosomal DNA fragmentation in HL-60 cells [43] Inhibiting melanogenesis and tyrosinase protein expression were 13 and 30μM, respectively [44] Anti-inflammatory effect on the affected brain [45] Reduce the hippocampal microglial activation induced by chronic infusions of lipopolysaccharide (LPS) into the fourth ventricle of Fisher-344 rat brains [46] Protect HepG2 cells against *tert*-butylhydroperoxide induced oxidative damage in a human hepatoma cell line [47] Protective effects on cisplatin-induced hepatotoxicity may be associated with the mitogen activated protein kinase (MAPK) signaling pathway [48] Enhanced insulin sensitivity and improved lipid metabolic disorders [49]
β-Sitosterol (**137**)	Antiserum, in neutralizing snake venom-induced actions [50] Cytotoxic [51, 52] - Bowes cells with IC_{50} 36.54 μM [51] - MCF7 with IC_{50} 357.51 μM [52] and 72 μM [52] - KB with $IC_{50} > 100$ μM [52] - CasKi with IC_{50} 62 μM [52]

Table 3.1 (Continued)

Compounds	Activities [References]
β-Sitosterol (**137**)	- HCT 116 with $IC_{50} > 100$ μM [52] - A549 with IC_{50} 78 μM [51] - MRC-5 with $IC_{50} > 100$ μM [52]
Stigmasterol (**70**)	Antiserum, in neutralizing snake venom-induced actions [50]

The structures of all isolated compounds were identified by interpretation of their spectral data of IR, EIMS, ^{1}H and ^{13}C NMR spectra including DEPT, COSY, HMQC and HMBC experiments, as well as by comparison of their spectral data with those reported in the literature.

Lupeol palmitate (**134**); a triterpene called (3β)-lup-20(29)-en-3-yl palmitate in the systematic name, was isolated from fraction VS2 of CH_2Cl_2 extract of *V. scandens* aerial parts as a white solid, mp 83.5–84.0 °C that conform to the result in previous report shown between 80–81.5 °C [53] and analyzed for $C_{47}H_{83}O_2Na$ by means of HRMS measurement on the $[M+Na]^+$ ion (*m/z*) 687.5640 calcd for 687.6056. The key fragmentation ions in the mass spectrum of this compound at *m/z* 664 [M^+], 408 and 189 (base peak), were useful in obtaining the structure of **134**, was shown in Figure 3.4. The IR spectrum indicated three significant signals that consisted stretching of carbonyl at 1728, stretching of carbon of double bond at 1641, and stretching of ether bond in carboxylate group at 1173 cm^{-1}. The ^{1}H NMR spectrum of this compound displayed characteristic signals, for example, H-3 proton adhered on the C-3 carbon showed multiplet signal at 4.47 ppm. The H-19 proton, which adjoined C-19, displayed triplet of doublets signal at 2.38 ppm (J = 11.1, 5.7 Hz), which associated with tertiary carbon of terminal double bond outside ring E, and H-29 proton, methylene proton of the terminal double bond outside ring E, showed doublet of doublets signal peaks at 4.57 (J = 2.3, 1.3 Hz) and showed doublet at 4.68 ppm (J = 2.3 Hz).

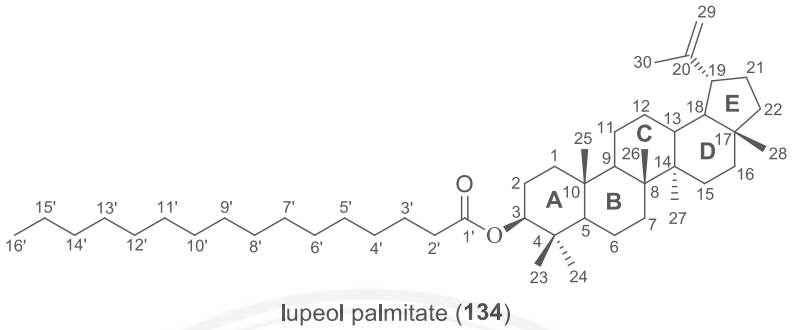


Figure 3.1 Labeling number of each carbon in structure of lupeol palmitate (**134**)

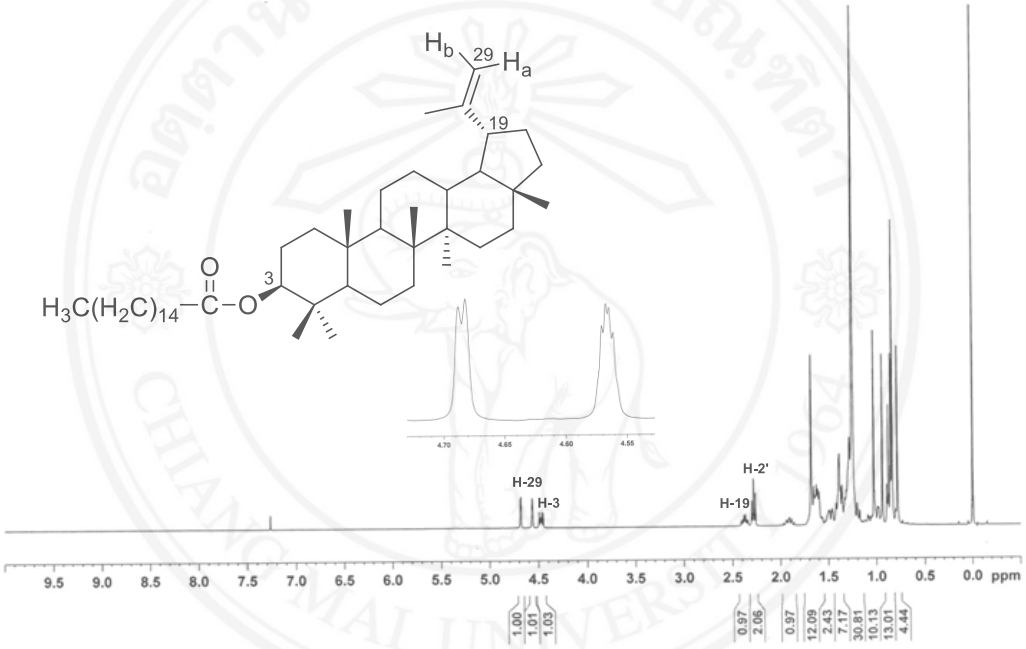


Figure 3.2 ^{1}H NMR spectrum of lupeol palmitate (**134**)

The ^{13}C NMR of this compound performed significant signals, for example, C-3 carbon that bonded with carboxylate group showed peak at 80.6 ppm, a quarternary carbon (C-20) displayed peak at 150.9 ppm, a methylene carbon of terminal double bond (C-29) displayed signal at 109.4 ppm, C-1′ carbon identified as carbonyl carbon of carboxylate group showed evident signal at 173.6 ppm, and C-2′ to 16′ carbons, side chain of alkyl branch of carboxylate group, demonstrated in the range of 29.2–29.8 ppm. In combination with DEPT-135 and DEPT-90 spectra, these signals led to the possible classification of the 46 carbons as shown below;

$8 \times CH_3$	at δ	14.1, 14.5, 16.0, 16.2, 16.6, 18.0, 19.3, 28.0
$25 \times CH_2$	at δ	18.2, 20.9, 22.7, 23.7, 25.1, 25.2, 27.4, 29.2–29.8, 31.9, 34.2, 34.8, 35.6, 38.4, 38.4, 40.0, 109.4
$6 \times CH$	at δ	38.0, 48.0, 48.3, 50.3, 55.4, 80.6
$7 \times C$	at δ	37.1, 37.8, 40.8, 42.8, 43.0, 150.9, 173.6.

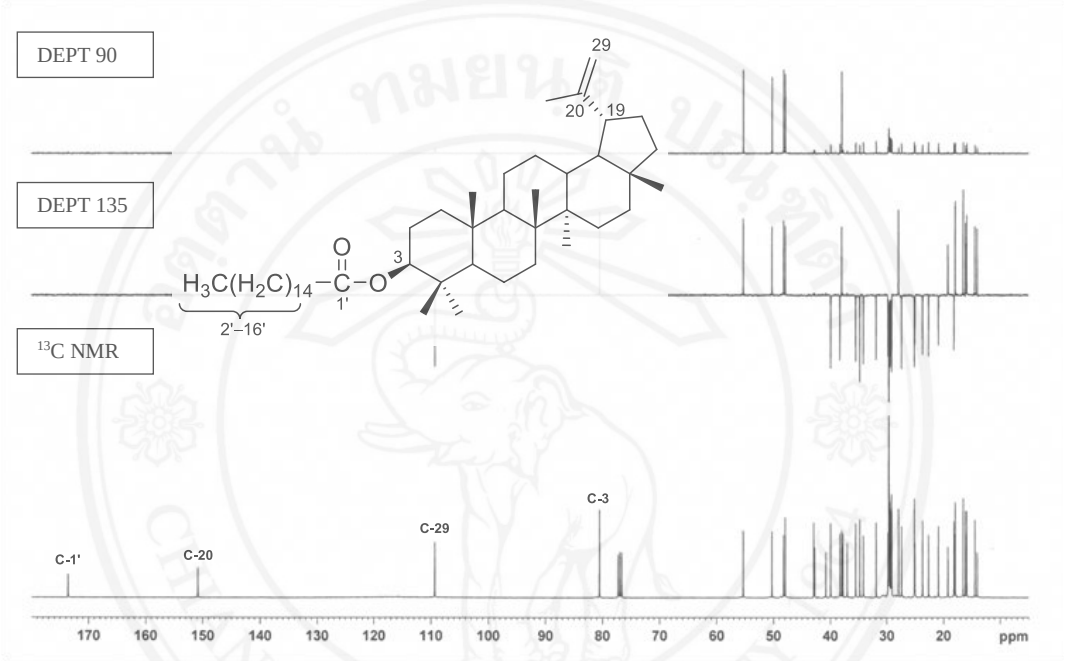


Figure 3.3 DEPT 90, DEPT 135 and ^{13}C NMR spectra of lupeol palmitate (**134**)

The correlation between ^{1}H and ^{13}C were confirmed by HMQC spectrum. From this data, H-3 proton was coupled with C-3 carbon, H-19 which accorded with C-19, two protons (H-29) of methylene group correlated with C-29 carbon, the methylene protons (H-2′ protons) of long chain were linked with C-2′ carbon. Therefore, we could deduce all protons, which were identified, were bonded with all correlated carbon. All spectral data of lupeol palmitate (**134**) were shown in Table 3.2 and 3.3.

To confirm the evidence, HMBC spectrum, explained correlation between proton and carbon such as proton 3 on carbon that linked with palmitate chain (C-3) with the carbonyl carbon C-1′ as well as associated with carbon 2, 4, 23 and 24. Further the signal of proton 29, outside ring E, showed correlation with carbon 19, 20 and 30. The spectroscopic data of compound **134** was found to be in agreement with those of lupeol

palmitate, reported by Kui-Wu, W., 2007 [52] and isolated from the leaves of *Viburnum urceolatum* [54] and deertongue [55].

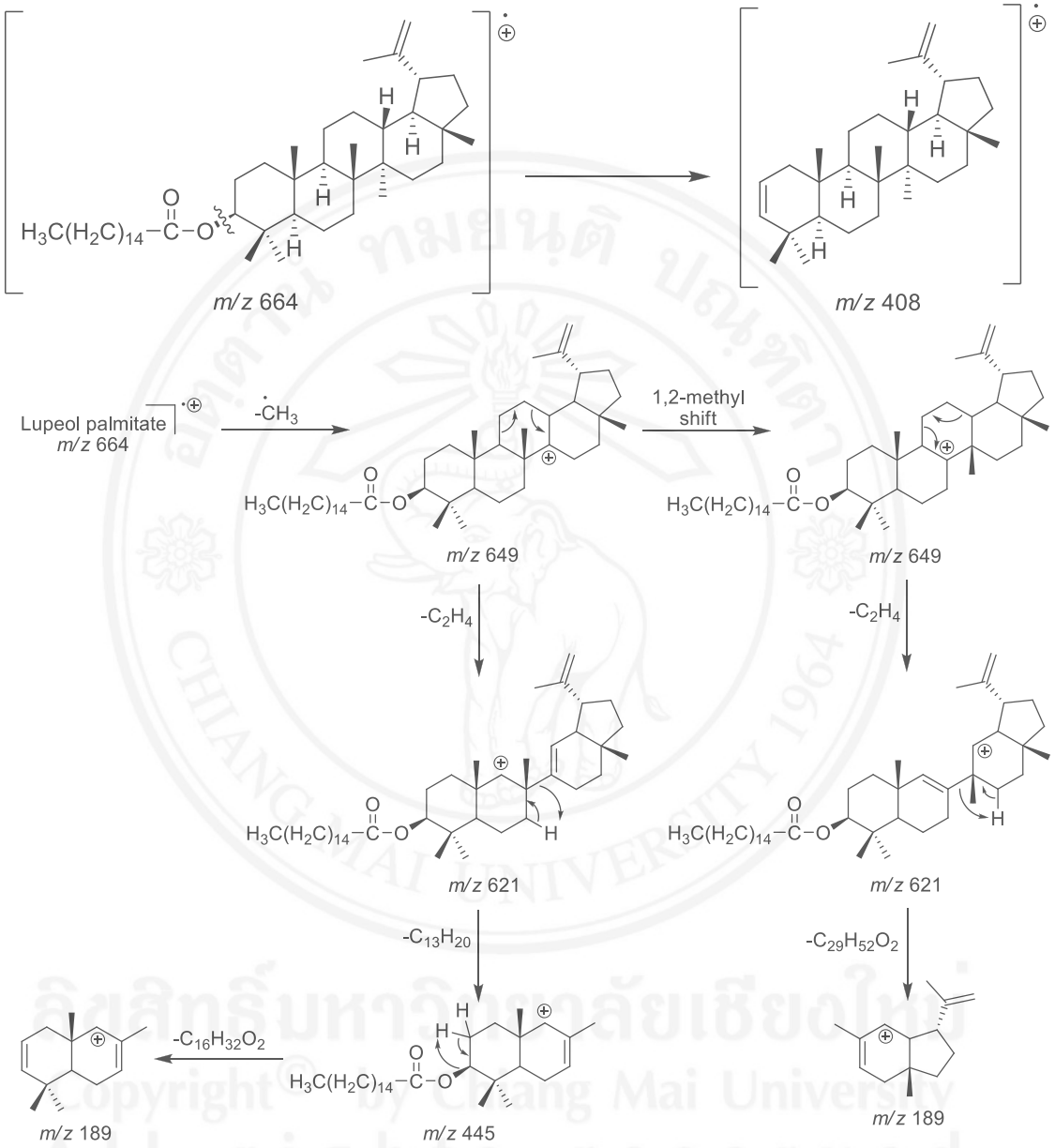


Figure 3.4 Mechanism of key fragment ions of lupeol palmitate (**134**) from EIMS

Table 3.2 ^{1}H and ^{13}C NMR data of lupeol palmitate (**134**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [54] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [53] (ppm)
1			38.4	38.5
2			23.7	23.7
3	4.47 (*m*)	4.33 (*m*)	80.6	80.6
4	-	-	37.8	39.5
5			55.4	55.4
6			18.2	18.3
7			34.2	32.7
8	-	-	40.8	40.6
9			50.3	50.4
10	-	-	37.1	37.4
11			20.9	21.6
12			25.2	26.5
13			38.0	35.5
14	-	-	42.8	42.6
15			27.4	27.5
16			35.6	35.6
17	-	-	43.0	45.9
18			48.3	48.5
19	2.38 (*td*, 11.1, 5.7)		48.0	47.8
20	-	-	150.9	150.9
21			38.4	39.5
22			40.0	39.9
23	0.84 (*s*), 0.88 (*s*), 0.86 (*s*)	0.81, 0.86, 0.96	28.0	28.3
24			16.0	15.6
25			16.6	16.3
26	1.03 (*s*)	1.06, 1.29	16.2	15.9
27	0.94 (*s*)		14.5	14.6
28	0.78 (*s*)		18.0	18.5
29a	4.57 (*dd*, 2.3, 1.3) or 4.68 (*d*, 2.3)	4.58 (*m*)	109.4	109.5

Table 3.2 (Continued)

Positions	δ_H (Mult., *J* in Hz)	δ_H [54] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [53] (ppm)
29b	4.57 (*dd*, 2.3, 1.3) or 4.68 (*d*, 2.3)	4.68 (*m*)	109.4	109.5
30	1.68 (*s*)	1.68 (*s*)	19.3	19.6
1′	-	-	173.6	173.7
2′	2.27 (*t*, 7.2)		34.8	34.9
3′			25.1	25.2
4′–13′			29.2–29.8	29.4–29.9
14′			31.9	32.1
15′			22.7	22.8
16′	0.88 (*t*)		14.1	14.2

Recorded in $CDCl_3$

Table 3.3 ^1H-^1H COSY, HMQC, HMBC data of lupeol palmitate (**134**) ($CDCl_3$) at 400 MHz (^1H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^1H-^1H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
3	H-2	C-3	C-2, 4, 23, 24, 1′
19	H-18, 21	C-19	C-18, 20, 21, 29, 30
23	-	C-23	C-3, 4, 5, 24
24	-	C-24	C-3, 4, 23
25	-	C-25	C-1, 5, 9, 10
26	-	C-26	C-8, 9
27	-	C-27	C-8, 13, 14, 15
28	-	C-28	C-16, 17, 18, 22
29	-	C-29	C-19, 20, 30
30	-	C-30	C-19, 20, 29
2′	H-3′	-OCO$\underline{C}H_2(CH_2)_{13}CH_3$	C-1′, 3′, 4′
16′	H-15′	-OCO$(CH_2)_{14}\underline{C}H_3$	C-14′, 15′

Recorded in $CDCl_3$

Lupeol acetate (**135**); called lup-20(29)-en-3-yl acetate in the systematic name, was divided from the same extract of **134** by comprehensive column chromatography. It was obtained as a white solid, mp 210.0–212.0 °C (from EtOAc/hexane) compared with mp 190–192 °C from the previous study [36] and analyzed for $C_{32}H_{52}O_2Na$ by means of HRMS measurement on the $[M+Na]^+$ ion *m/z* 491.3865 calcd for 491.3863. From EI-MS technique that received the molecular ion peak at *m/z* 468 $[M^+]$. The other key fragments were noticed and recorded at *m/z* 408 and 189, which is similar to the breakdown of lupeol palmitate as shown in Figure 3.8. Measuring of IR spectrum of this compound indicated three significant signals that consisted stretching of carbonyl at 1731, stretching of carbon double bond at 1641, and stretching of ether bond in carboxylate group at 1249 cm^{-1}. The ^1H NMR spectrum of this compound **135** was similar to compound **134** with the only difference at the absence of long chain protons signals. Oxymethine proton H-3 adhered on the C-3 carbon showed doublet of doublets signal (J = 10.4, 5.4 Hz) at 4.47 ppm and H-19 proton adhered on C-19 carbon, which associated with quarternary carbon of terminal double bond outside ring E, displayed triplet of doublets signal (J = 11.1, 5.7 Hz) at 2.38 ppm. In addition, olefinic protons of C-29 outside ring E showed two signal at 4.56 ppm that was doublet of doublets (J = 2.3, 1.3 Hz) and 4.68 ppm which was doublet (J = 2.3 Hz), and H-2′ was identified as three protons of methyl group showed signal of acetyl group at 2.04 ppm (Figure 3.6 and Table 3.4).

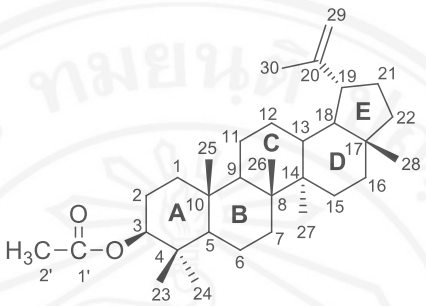
Their ^{13}C NMR spectrum also showed especial signals, for example, C-3 carbon that adjoined acetate carbon indicated peak at 80.9 ppm, C-20 carbon was identified as quarternary carbon of double bond outside ring E showed peak at 150.9 ppm, C-29 carbon, which was methylene carbon of terminal double bond, displayed signal at 109.3 ppm, and C-1′ carbon, carbonyl carbon of acetate group, showed obvious peak at 171.0 ppm. In combination with DEPT-135 and DEPT-90 spectra, these signals led to the possible classification of the 32 carbons as shown below;

$8 \times CH_3$ at δ 14.5, 15.9, 16.1, 16.4, 17.9, 19.2, 21.6, 27.9

$11 \times CH_2$ at δ 18.1, 20.9, 23.7, 25.0, 27.4, 29.8, 34.2, 35.5, 38.3, 39.9, 109.3

$6 \times CH$ at δ 38.0, 48.0, 48.2, 50.3, 55.4, 80.9

$7 \times C$ at δ 37.0, 37.7, 40.8, 42.8, 42.9, 150.9, 171.0.



lupeol acetate (**135**)

Figure 3.5 Labeling number of each carbon in structure of lupeol acetate (**135**)

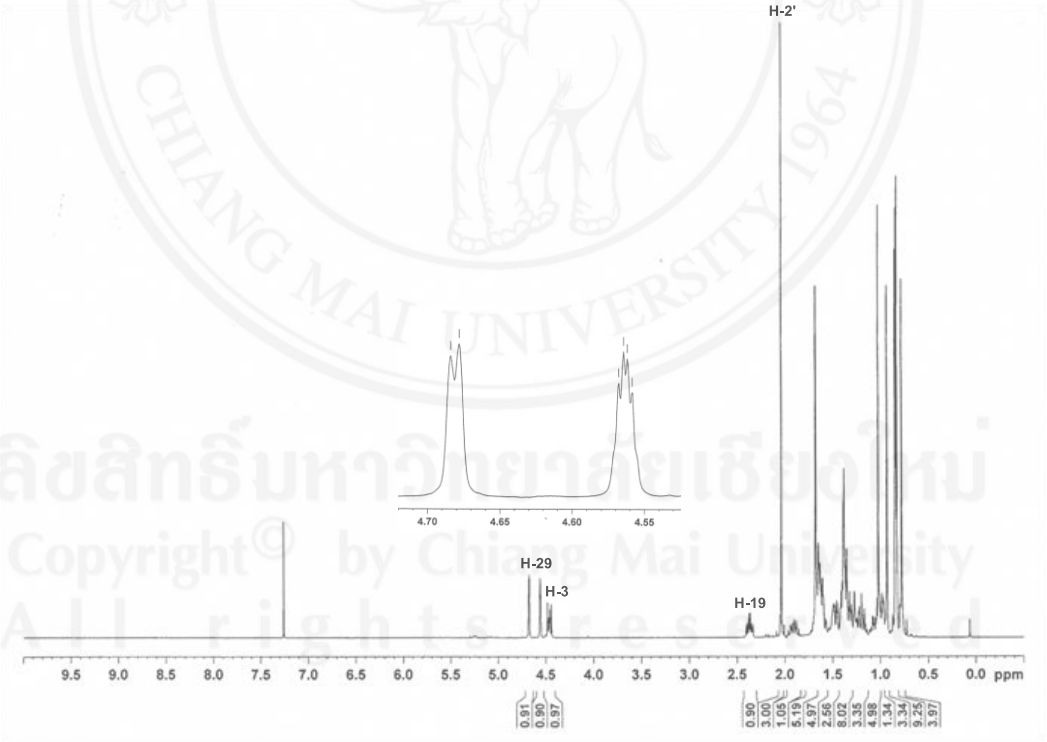


Figure 3.6 ^{1}H NMR spectrum of lupeol acetate (**135**)

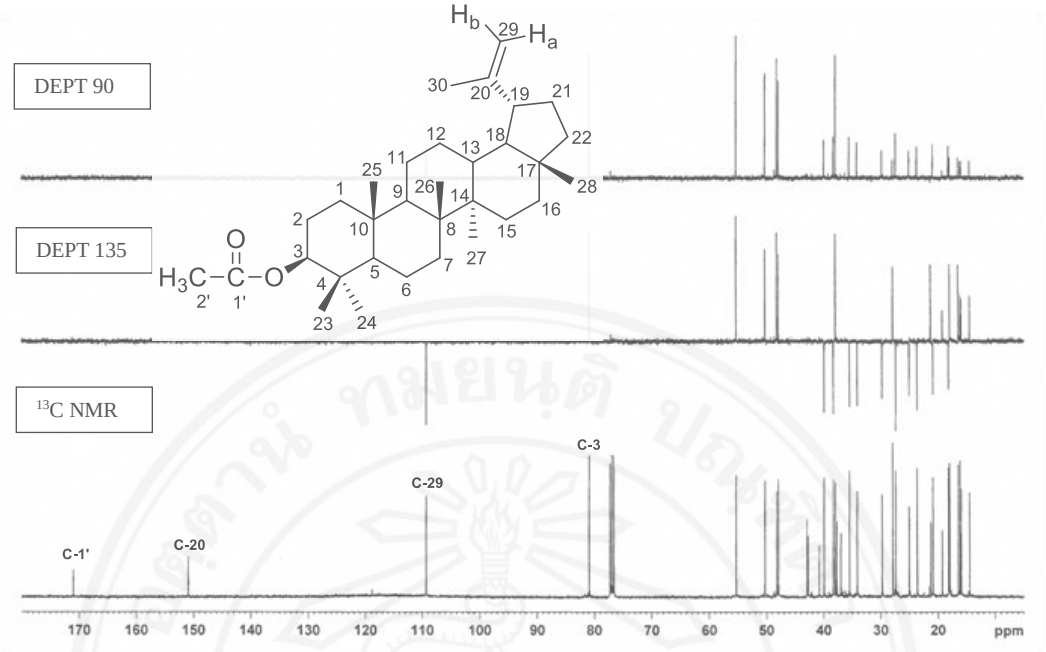


Figure 3.7 DEPT 90, DEPT 135 and ^{13}C NMR spectra of lupeol acetate (**135**)

Moreover, this compound was approved the structure by HMQC data that indicates the correlation between each proton and carbon. For instance, H-3 proton at 4.47 ppm coupled with C-3 at 80.9 ppm, H-19 proton at 2.37 ppm accorded with C-19 at 48.2 ppm, H-29 protons, two protons of methylene group, at 4.56 and 4.68 ppm correlated with C-29 at 109.3 ppm, and H-2′ protons, three protons of methyl group of acetate group, at 2.04 ppm correlated with C-2′ at 21.6 ppm, respectively.

The HMBC spectrum showed correlation of proton signal at δ 4.47 (H-3) with the carbonyl at δ 171.0 (C-1′) as well as with the signal at δ 15.9 (C-24) and 27.9 (C-23). Further the signal of olefinic proton at δ 4.56 and 4.68 (H-29) showed correlation with the signal at δ 19.2 (C-30) and 48.2 (C-19). By further analysis of 1D and 2D data and by comparison of the spectroscopic data with those of the literature values, the structure of **135** was elucidated as lupeol acetate. This compound was also been isolated from *Diospyros rubra* lec [36] and *Phyllanthus reticulates* [56].

Table 3.4 ^{1}H and ^{13}C NMR data of lupeol acetate (**135**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [36] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [36] (ppm)
1			38.3	38.3
2			23.7	23.7
3	4.47 (*dd*, 10.4, 5.4)	4.44 (*dd*, 10.8, 5.8)	80.9	80.9
4	-	-	37.7	37.7
5	0.78 (*m*)	0.76 (*dd*, 10.8, 5.8)	55.4	55.3
6			18.1	18.1
7			34.2	34.2
8	-	-	40.8	40.8
9			50.3	50.3
10			37.0	37.0
11			20.9	20.9
12			25.0	25.0
13			38.0	38.0
14	-	-	42.8	42.8
15			27.4	27.4
16			35.5	35.5
17	-	-	42.8	42.9
18			48.0	48.0
19	2.38 (*td*, 11.1, 5.7)	2.33 (*dt*, 11.1, 5.6)	48.2	48.2
20	-	-	150.9	150.9
21	1.86-1.96 (*m*)	1.82-1.93 (*m*)	29.8	29.8
22			39.9	39.9
23	0.84 (*s*)	0.82 (*s*)	27.9	28.2
24	0.84 (*s*)	0.82 (*s*)	15.9	15.9
25	0.84 (*s*)	0.82 (*s*)	16.4	16.1
26	1.02 (*s*)	1.00 (*s*)	16.1	16.4
27	0.93 (*s*)	0.91 (*s*)	14.5	14.5
28	0.83 (*s*)	0.81 (*s*)	17.9	17.9

Table 3.4 (Continued)

Positions	δ_H (Mult., *J* in Hz)	δ_H [36] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [36] (ppm)
29a	4.56 (*dd*, 2.3, 1.3) or 4.68 (*d*, 2.3)	4.54 (*brs*)	109.3	109.3
29b	4.56 (*dd*, 2.3, 1.3) or 4.68 (*d*, 2.3)	4.66 (*brs*)	109.3	109.3
30	1.68 (*s*)	1.66 (*s*)	19.2	19.0
1′	-	-	171.0	171.0
2′	2.04 (*s*)	2.01 (*s*)	21.6	21.3

Recorded in $CDCl_3$

Table 3.5 ^1H-^1H COSY, HMQC, HMBC data of lupeol acetate (**135**) ($CDCl_3$) at 400MHz (^1H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^1H-^1H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
3	H-2	C-3	C-2, 4, 23, 24, 1′
19	H-18, 21	C-19	C-18, 20, 21, 29, 30
23	-	C-23	C-3, 4, 5, 24
24	-	C-24	C-3, 4, 23
25	-	C-25	C-1, 5, 9, 10
26	-	C-26	C-8, 9
27	-	C-27	C-8, 13, 14, 15
28	-	C-28	C-16, 17, 18, 22
29	-	C-29	C-19, 20, 30
30	-	C-30	C-19, 20, 29
2′	H-3′	-OCO$\underline{C}H_2(CH_2)_{13}CH_3$	C-1′, 3′, 4′
16′	H-15′	-OCO$(CH_2)_{14}\underline{C}H_3$	C-14′, 15′

Recorded in $CDCl_3$

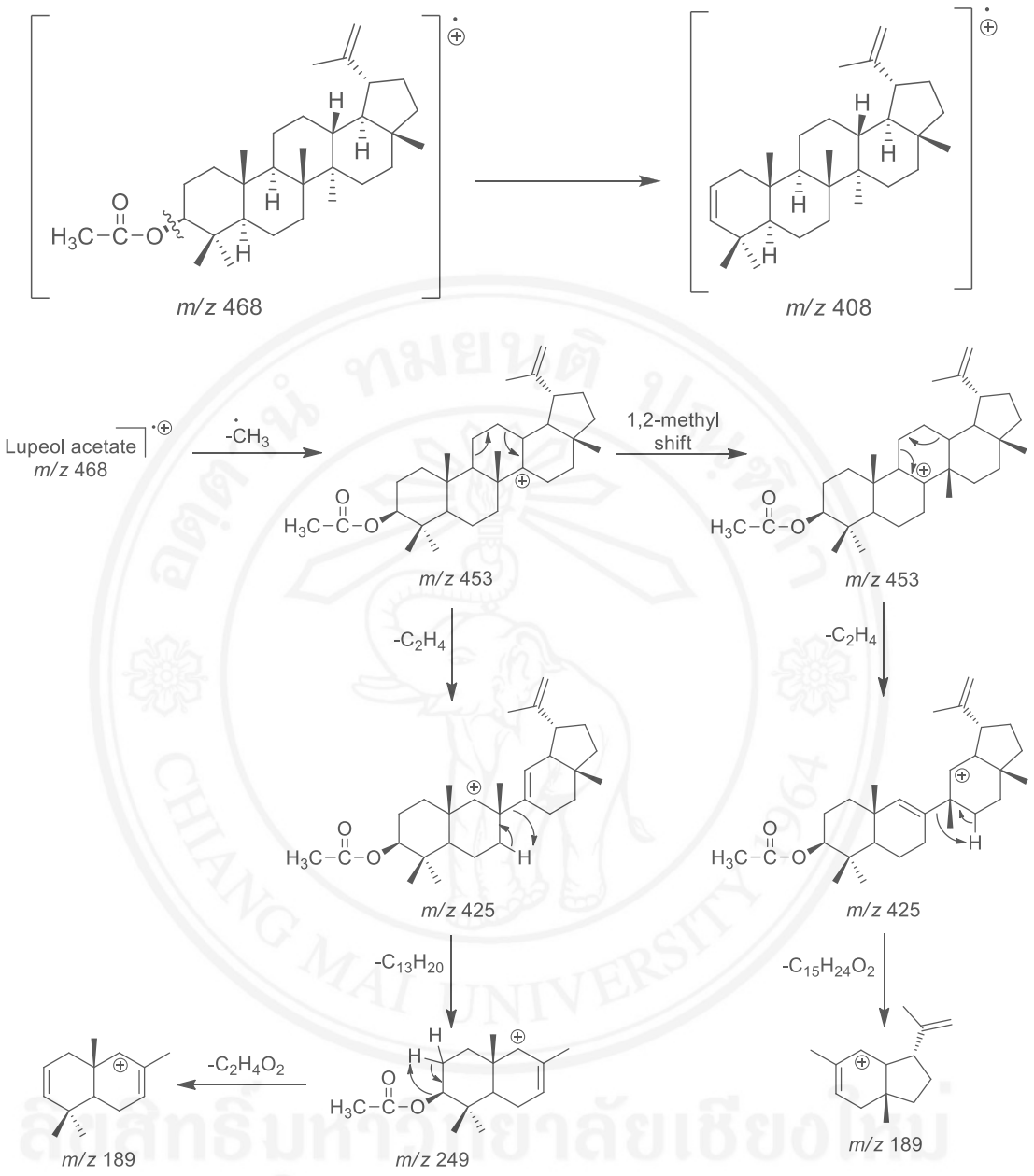
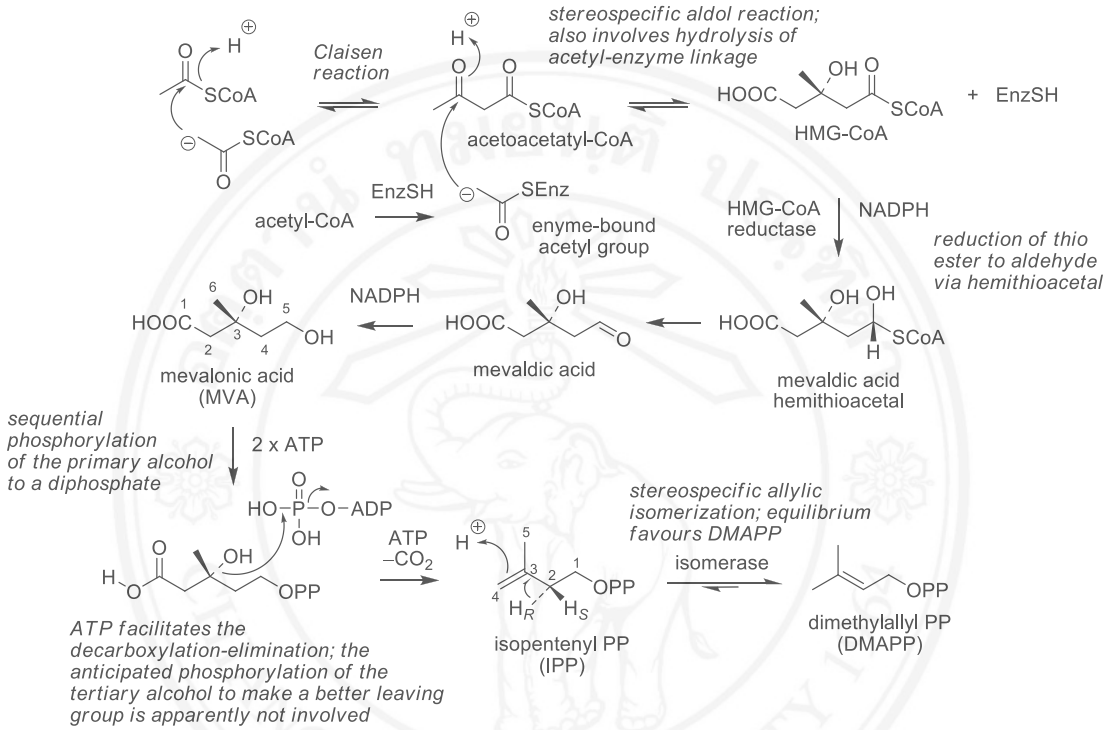
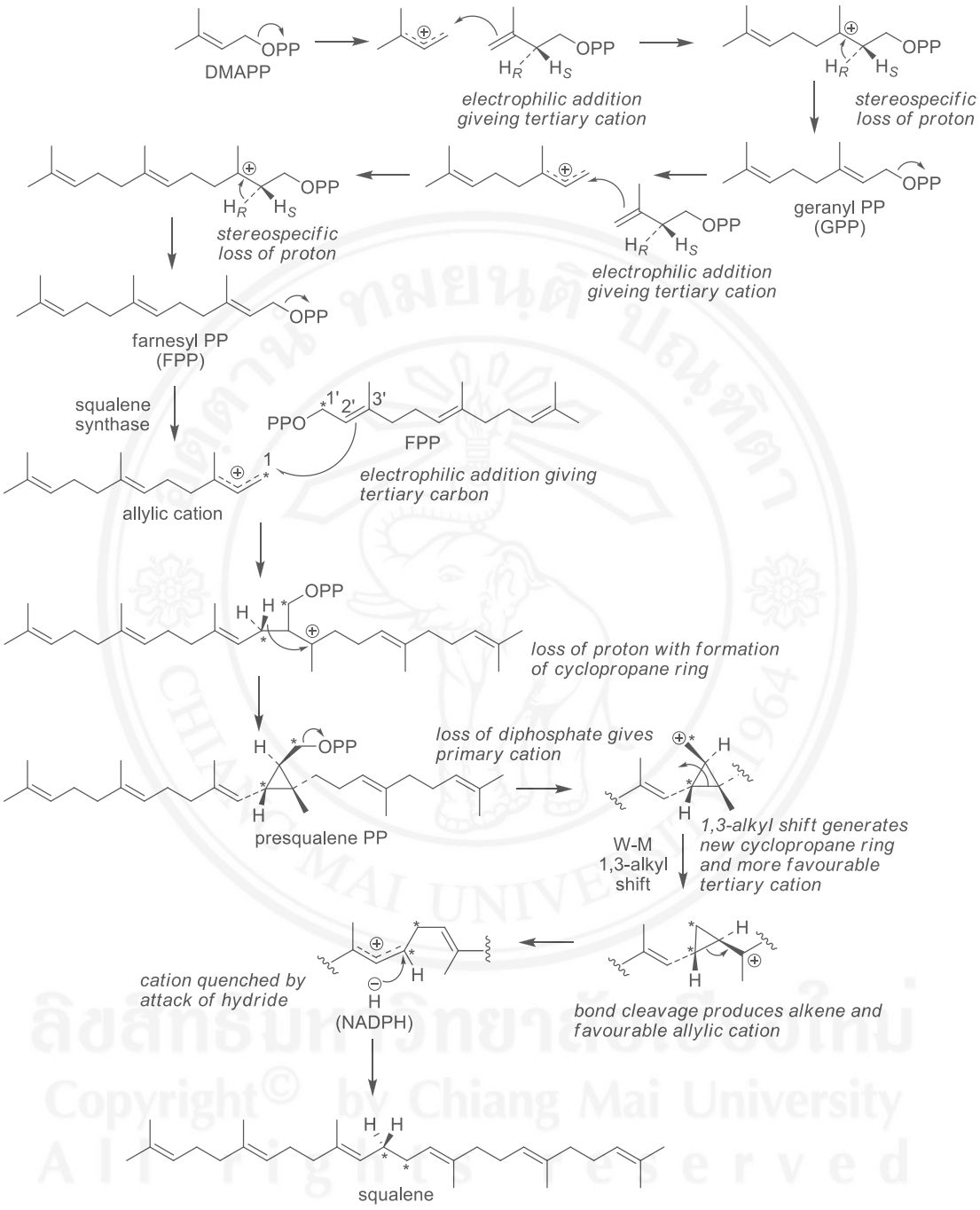


Figure 3.8 Mechanism of key fragment ions of lupeol acetate (**135**) from EIMS

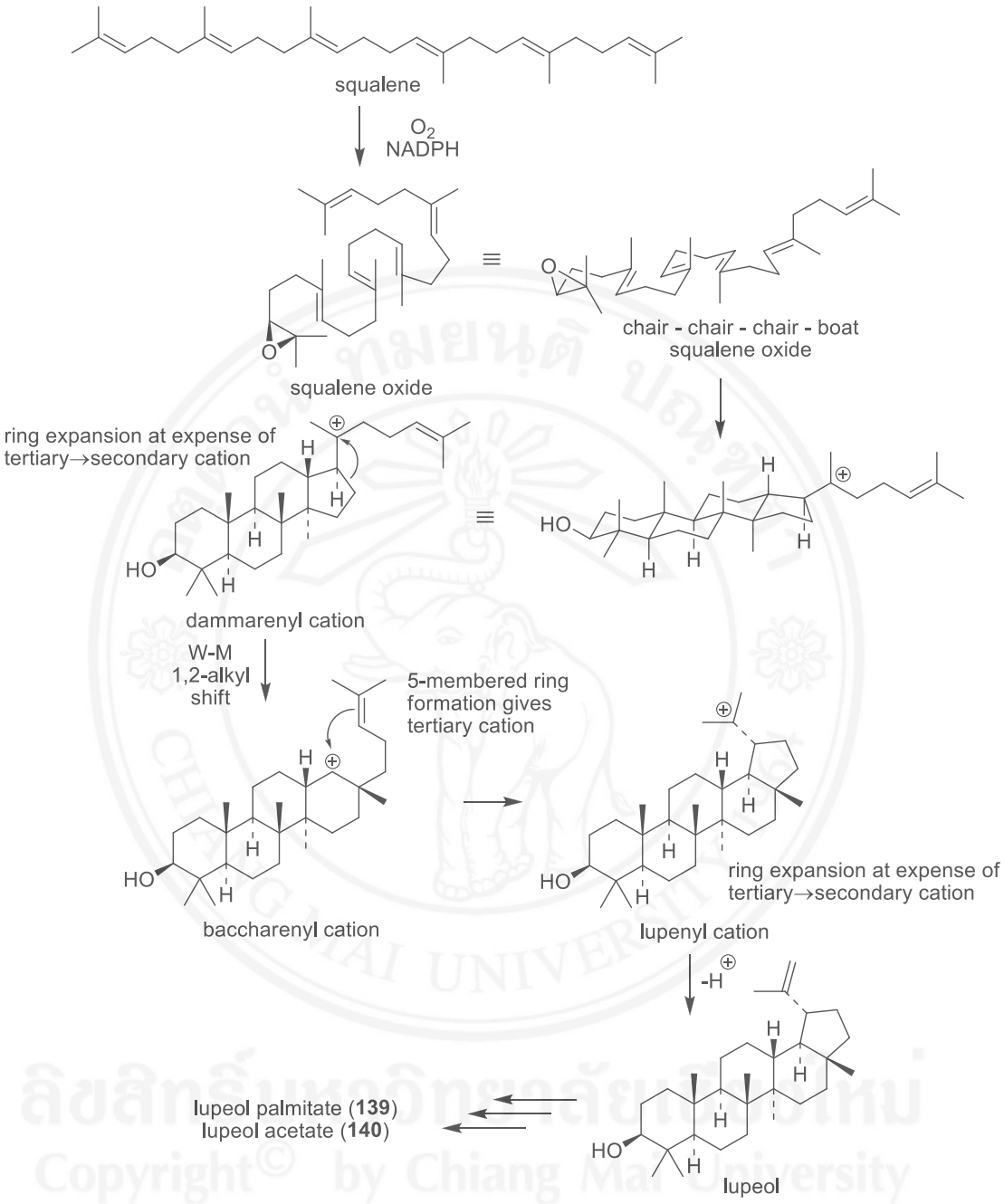
The two triterpenes, lupeol palmitate (**134**) and lupeol acetate (**135**), which were isolated from *V. scandens*, were well known compounds which has biosynthesis from Mavalonate pathway [57]. The biosynthetic mechanism of triterpens is shown in Scheme 3.1.



Scheme 3.1 Biosynthesis of triterpenes *via* Mevalonate pathway



Scheme 3.1 (Continued)

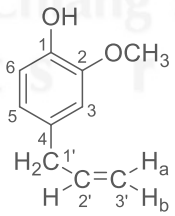


Scheme 3.1 (Continued)

Eugenol (**98**); 4-allyl-2-methoxyphenol for systematic name, was obtained as a yellow oils and isolated from fraction VS4 of CH_2Cl_2 extract of *V. scanden* aerial parts. The HRMS (positive ion mode) exhibited a pseudomolecular ion peak at (*m/z*) 187.0736 $[M+Na]^+$ ion calcd for 187.0735, corresponding to a molecular formula $C_{10}H_{12}O_2Na$, and exhibited a molecular ion peak in EIMS at *m/z* 164 $[M]^+$ (positive ion mode) which agreed with the molecular formula of $C_{10}H_{12}O_2$. Fragmentation of eugenol (**98**) was indicated in Figure 3.12 compared with previous study [58, 59]. The IR spectrum exhibited hydroxyl group at 3506 cm^{-1}, alkene C=C and aromatic ring at 1610 cm^{-1}, and C-O stretching band around 1268 cm^{-1} present in eugenol. The ^1H NMR (400 MHz) spectrum of compound **98** in $CDCl_3$ showed signal of substituted aromatic ring at δ 6.69 (1H, *m*, H-3,5) and 6.85 (1H, *m*, H-6) could be assigned as 1,2,4-trisubstituted benzene, broad single proton of hydroxyl group at δ 5.50, and singlet signals of one methoxy group appeared at 3.88 ppm. The multiplet signals on C-3′, indicating methylene proton of terminal double bond at δ 5.07, confirmed by ^1H-^1H COSY spectrum of the H-2′ and H-3′.

The ^{13}C NMR displayed 10 carbon atoms which are corresponding to DEPT-135 and DEPT-90 spectra, these signals led to the possible classification of the 10 carbons as shown below;

$1 \times CH_3$	at δ	55.8
$2 \times CH_2$	at δ	39.8, 115.4
$4 \times CH$	at δ	111.1, 114.2, 121.1, 137.8
$3 \times C$	at δ	131.8, 143.8, 146.4.



eugenol (**98**)

Figure 3.9 Labeling number of each carbon in structure of eugenol (**98**)

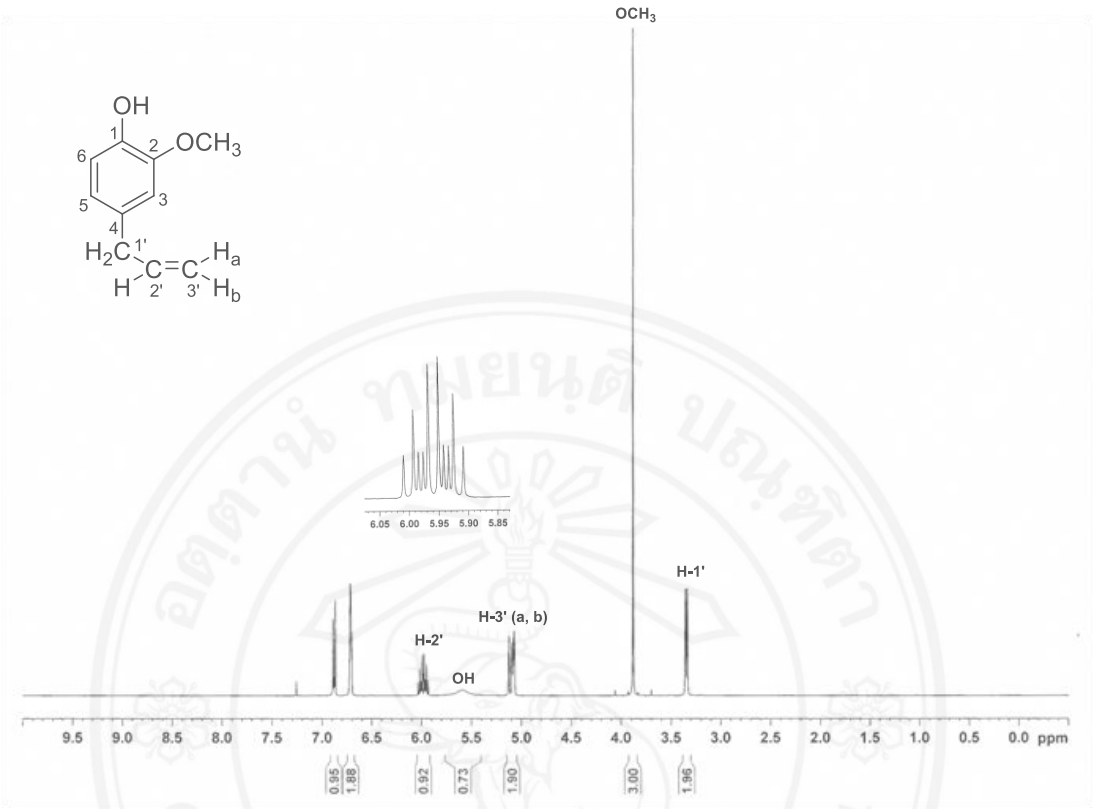


Figure 3.10 ^{1}H NMR spectrum of eugenol (**98**)

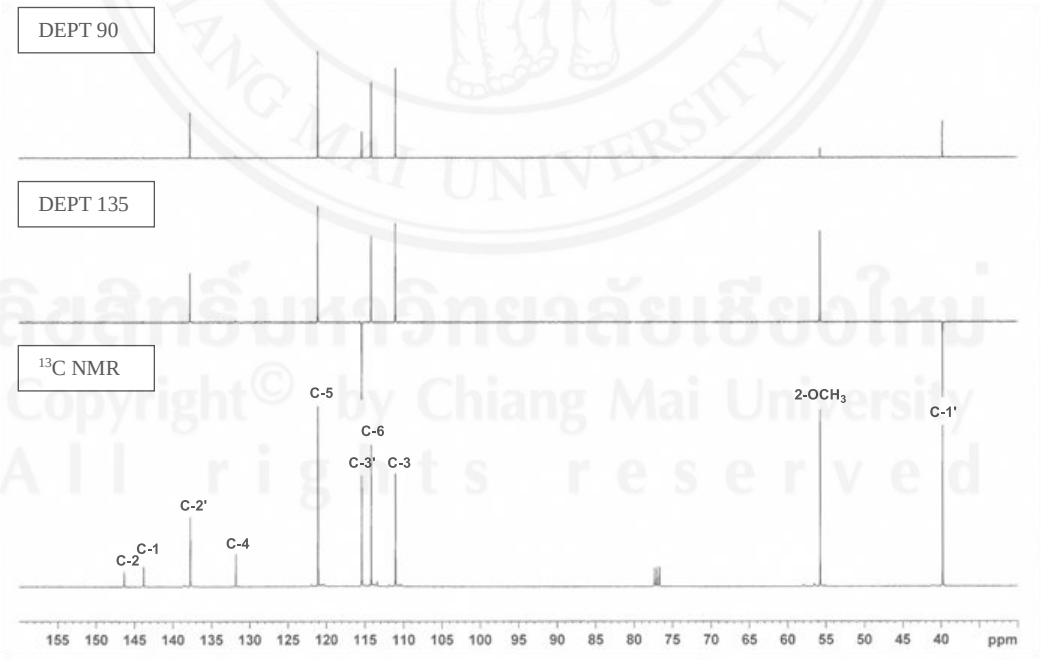


Figure 3.11 DEPT 90, DEPT 135 and ^{13}C NMR spectra of eugenol (**98**)

The HMBC spectrum showed correlation of proton signal at δ 3.23 (H-1′) with the methylene carbon (C-3′), methine carbon (C-5 and C-2′) and quaternary carbon (C-4) at δ 115.4, 121.1, 137.8, and 131.8 ppm, respectively. Further the signal of olefinic proton at δ 5.07 (H-3′) showed correlation with the signal at δ 39.8 (C-1′) and 137.8 (C-2′), and methoxy proton at 3.88 ppm correlated with quaternary carbon C-2 at 146.4 ppm. Therefore, these spectroscopic data was elucidated the structure of compound **98** as eugenol. Our assignment was found to be consistent with those reported by Anuj *et al.*, 2010 [60] and Emanuela *et al.*, 2004 [61].

Table 3.6 ^{1}H and ^{13}C NMR data of eugenol (**98**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [60] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [61] (ppm)
1	-	-	143.8	144.5
2	-	-	146.4	147.2
3	6.69 (*m*)	6.683	111.1	111.8
4	-	-	131.8	132.2
5	6.69 (*m*)	6.683	121.1	121.5
6	6.85 (*m*)	6.824	114.2	115.3
1′	3.32 (*d*, 6.7)	3.305	39.8	40.1
2′	5.96 (*ddt*, 16.9, 10.1, 6.7)	5.902	137.8	138.5
3′	5.07 (*m*)	5.034	115.4	115.3
1-OH	5.50 (*brs*)	5.902	-	-
2-OCH_3	3.88 (*s*)	3.871	55.8	55.8

Recorded in $CDCl_3$

Table 3.7 ^{1}H-^{1}H COSY, HMQC, HMBC data of eugenol (**98**) ($CDCl_3$) at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^{1}H-^{1}H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
3	-	C-3	C-1, 2, 4, 5, 1′
5	H-6	C-5	C-1, 3, 4, 6, 1′
6	H-5	C-6	C-1, 2, 4, 5
1′	H-2′	C-1′	C-3, 4, 5, 2′, 3′
2′	H-1′, 3′	C-2′	C-4, 1′
3′	H-2′	C-3′	C-1′, 2′

Recorded in $CDCl_3$

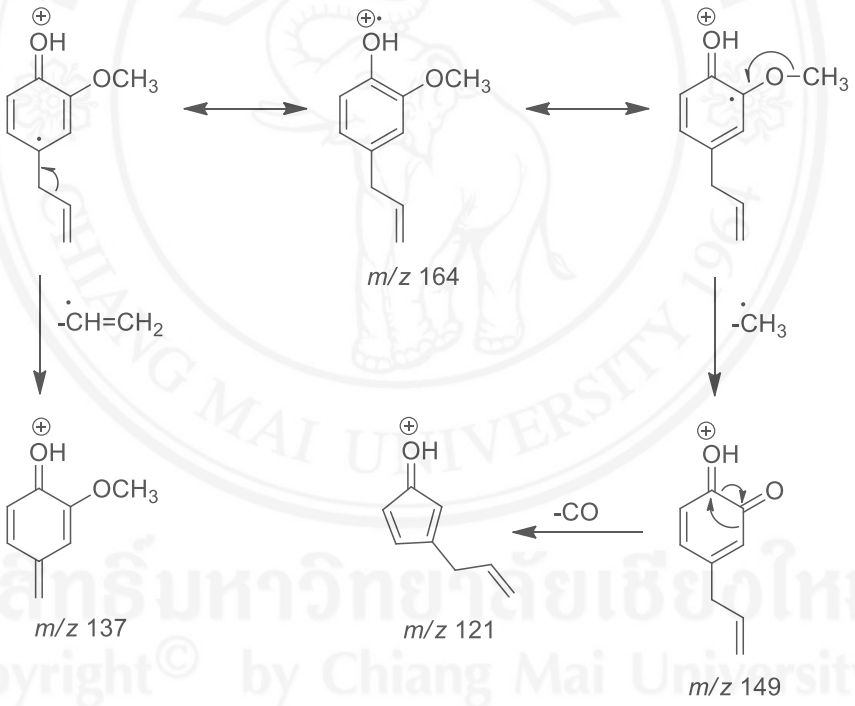
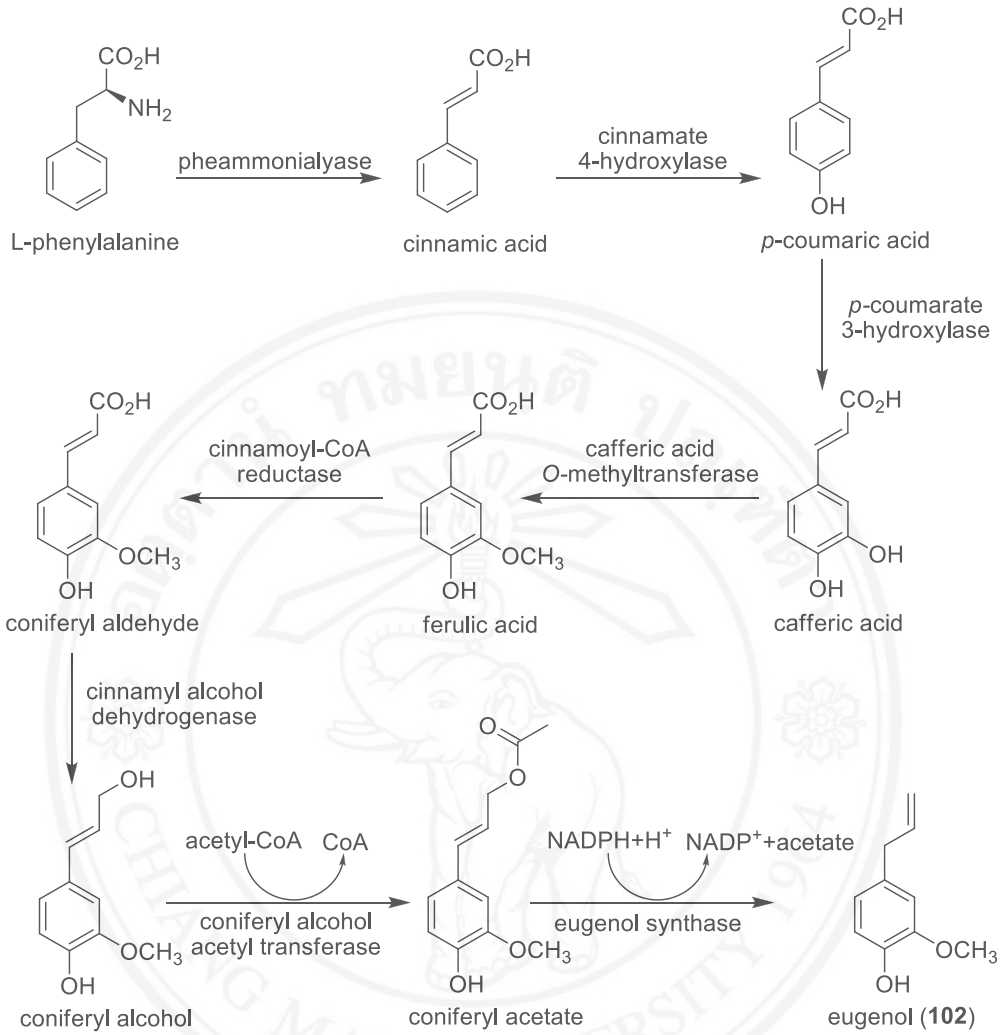


Figure 3.12 Mechanism of key fragment ions of eugenol (**98**) from EIMS

Eugenol (**98**), as phenylpropene make up the bulk of plant volatile oils, synthesized *via* the phenylalanine and coniferyl alcohol as depicted in Scheme 3.2 [62, 63].



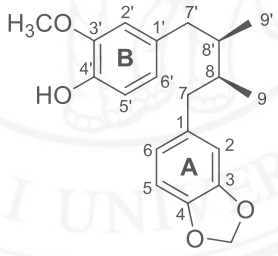
Scheme 3.2 Biosynthesis of eugenol (**98**) *via* Shikimate pathway

Macelignan (**136**); 7-(3,4-methylenedioxyphenyl)-7′-(4′-hydroxy-3′-methoxy-phenyl)-8,8′-lignan for a systematic name, was obtained as a yellow gum from fraction VS5 of the CH_2Cl_2 extract of *V.* scandens aerial parts. From measuring optical rotation of compound, the value was observed and recorded as $[\alpha]_D^{27.5}$ = +7.8 (*c* 0.32, $CHCl_3$), whereas the reference was reported as $[\alpha]_D^{20}$ = +5.28 (*c* 1.8, $CHCl_3$) [64] and the IR spectrum showed the presence of hydroxyl group at 3538, alkene C=C and aromatic ring at 1608, and methylenedioxy group at 933 cm^{-1}. Its HRMS revealed a molecular ion at *m/z* 351.1498 $[M+Na]^+$ (calcd. 351.1573), which was consistent with an elemental formula of $C_{20}H_{24}O_4Na$. The EIMS displayed a $[M]^+$ at *m/z* 328 ($C_{20}H_{24}O_4$) and fragments at *m/*z 137 and 135, were useful in obtaining the structure of **136** as shown in

Figure 3.16. The NMR data of **136** clearly showed that the piperonyl group (ring A) give rise to an ABX system of proton signals at δ 6.73 (*d*, *J* = 7.9 Hz, H-5), 6.61 (*dd*, *J* = 8.2, 1.5 Hz, H-6) and 6.66 (*d*, *J* = 1.6 Hz, H-2). In addition, three protons of aromatic ring at δ 6.83 (*d*, *J* = 7.9 Hz, H-5′), 6.62 (*d*, *J* = 1.5 Hz, H-2′) and 6.65 (*dd*, *J* = 8.2, 1.6 Hz, H-6′), implying that the aromatic rings each had a 1,2,4-trisubstiution pattern (Figure 3.14 and Table 3.8).

The ^{13}C NMR spectrum of compound **136** also supported the presence of four oxygenated aromatic carbons at δ 147.4 (C-3), 145.4 (C-4), 146.3 (C-3′) and 143.5 (C-4′). In combination with DEPT-135 and DEPT-90 spectra, these signals led to the possible classification of the 20 carbons as shown below;

3 × CH_3	at δ	16.1, 16.2, 55.8
3 × CH_2	at δ	38.8, 39.0, 100.7
8 × CH	at δ	39.2, 39.3, 107.9, 109.3, 111.4, 114.0, 121.7, 121.8
6 × C	at δ	133.7, 135.7, 143.5, 145.4, 146.3, 147.4.



macelignan (**136**)

Figure 3.13 Labeling number of each carbon in structure of macelignan (**136**)

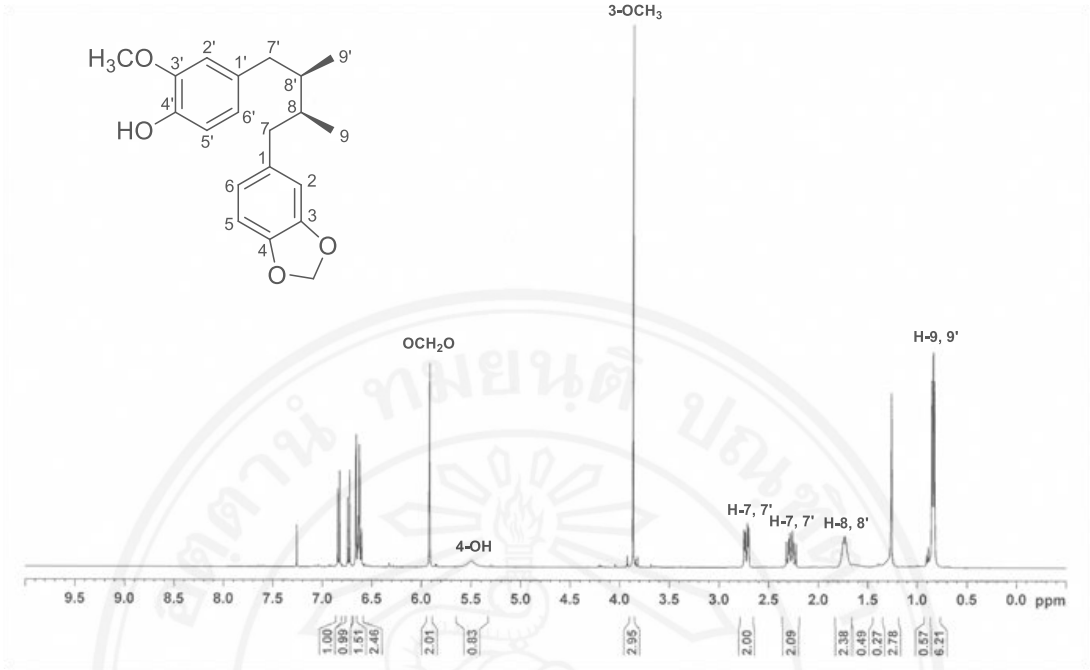


Figure 3.14 ^{1}H NMR spectrum of macelignan (**136**)

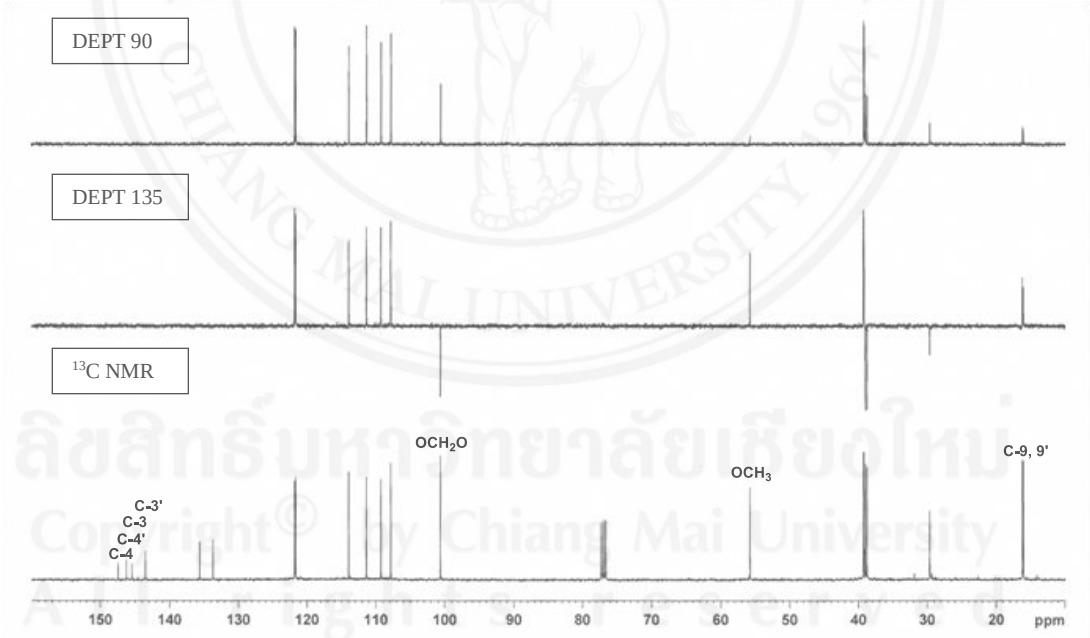


Figure 3.15 DEPT 90, DEPT 135 and ^{13}C NMR spectra of macelignan (**136**)

Furthermore the 2D-NMR spectra, HMQC and HMBC were recorded for this compound to confirm the assignment as shown in Table 3.9. In ^{1}H-^{13}C directed correlation observed in HMQC experiment verified that protons on C-8 and 8′ position at 1.74 ppm were correlated with C-8 and 8′ at 39.3 and 39.2 ppm, respectively, two protons on C-7 at 2.29 and 2.72 ppm had relationship with C-7, and two protons on C-7′ at 2.25 and 2.72 ppm correlated with C-7′ in the same way as proton on C-7 position. In addition, two protons at 5.92 ppm were bonded with carbon of diether group (OCH_2O), which adjoined with secondary carbons of ether group.

Moreover, the HMBC data, showed correlation of methylene proton signal at δ 5.92 ($OC\underline{H}_2O$) with the quaternary carbon (C-3 and 4) at δ 147.4 and 145.4 ppm, respectively. Furthermore, multiplets signal at δ 1.74 (*m*, H-8′) correlated with two methyl carbons C-9 and 9′ at δ 16.1 and 16.2 ppm, two methylene carbons C-7 and 7′ at δ 39.0 and 38.8 ppm and methine carbon C-8 at δ 39.3 ppm. Similar, proton H-8 can be correlated with two methyl carbons C-9 and 9′, two methylene carbons C-7 and 7′ and methine carbon C-8′ at δ 39.2 ppm. Further the methoxy proton signal at δ 3.86 showed correlations with the signal at δ 147.3 (C-3′) and 143.5 (C-4′). The spectroscopic data of compound **136** was found to be in agreement with those of macelignan, reported by Filleur *et al*., 2001 [65]. The comparisons of spectral data are provided in Table 3.8.

Table 3.8 ^{1}H and ^{13}C NMR data of macelignan (**136**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [65] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [65] (ppm)
1	-	-	135.7	135.7
2	6.66 (*d*, 1.6)	6.65 (*d*, 1.4)	109.3	109.4
3	-	-	147.4	147.5
4	-	-	145.4	145.5
5	6.73 (*d*, 7.9)	6.72 (*d*, 7.8)	107.9	107.9
6	6.61 (*dd*, 8.2, 1.5)	6.60 (*dd*, 7.8, 1.4)	121.7	121.7
7	2.29 (*dd*, 13.6, 9.1)	2.28 (*dd*, 13.6, 9.1)	39.0	39.1
	2.72 (*dd*, 13.6, 5.0)	2.72 (*dd*, 13.6, 5.0)		

Table 3.8 (Continued)

Positions	δ_H (Mult., *J* in Hz)	δ_H [65] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [65] (ppm)
8	1.74 (*m*)	1.73 (*m*)	39.3	39.4
9	0.83 (*d*, 6.6)	0.83 (*d*, 6.6)	16.1	16.1
1′	-	-	133.7	133.8
2′	6.62 (*d*, 1.5)	6.62 (*d*, 1.4)	111.4	111.4
3′	-	-	146.3	146.3
4′	-	-	143.5	143.6
5′	6.83 (*d*, 7.9)	6.82 (*d*, 7.8)	114.0	114.0
6′	6.65 (*dd*, 8.2, 1.6)	6.64 (*dd*, 7.8, 1.4)	121.8	121.8
7′	2.25 (*dd*, 13.6, 9.1)	2.25 (*dd*, 13.6, 9.1)	38.8	38.9
	2.72 (*dd*, 13.6, 5.0)	2.72 (*dd*, 13.6, 5.0)		
8′	1.74 (*m*)	1.73 (*m*)	39.2	39.3
9′	0.85 (*d*, 6.6)	0.84 (*d*, 6.6)	16.2	16.2
OCH_2O	5.92 (*dd*, 2.6, 1.4)	5.91 (*br d*, 1.3)	100.7	100.7
3-OCH_3	3.86 (*s*)	3.86 (*s*)	55.8	55.8
4-OH	5.50 (*brs*)	5.54 (*s*)	-	-

Recorded in $CDCl_3$

Table 3.9 ^{1}H-^{1}H COSY, HMQC, HMBC data of macelignan (**136**) ($CDCl_3$) at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^{1}H-^{1}H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
2	-	C-2	C-3, 4, 6, 7
5	H-6	C-5	C-1, 3, 4
6	H-5	C-6	C-2, 4, 5, 7
7	H-8	C-7	C-1, 2, 6, 8, 9, 8′
8	H-7, 9, 8′	C-8	C-7, 9, 7′, 8′, 9′
9	H-8	C-9	C-7, 8, 8′
2′	-	C-2′	C-3′, 4′, 6′, 7′
5′	H-6′	C-5′	C-1′, 3′, 4′, 6′

Table 3.9 (Continued)

Proton Position	^{1}H-^{1}H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
6′	H-5′	C-6′	C-1′, 2′, 4′, 5′, 7′
7′	H-8′	C-7′	C-8, 1′, 2′, 6′, 8′, 9′
8′	H-8, 7′, 9′	C-8′	C-7, 8, 9, 7′, 9′
9′	H-8′	C-9′	C-8, 7′, 8′
$OC\underline{H}_2O$	-	$OC\underline{C}H_2O$	C-3′, 4′
3-$OC\underline{H}_3$	-	3-$OC\underline{C}H_3$	C-3, 4

Recorded in $CDCl_3$

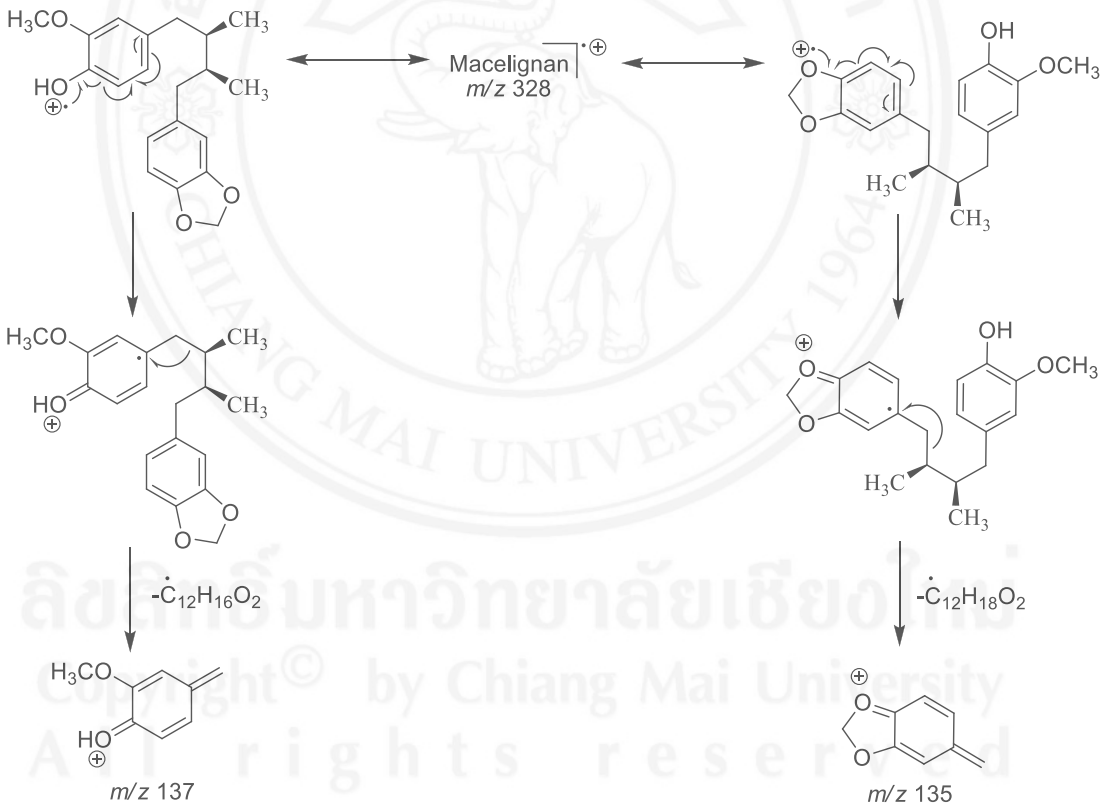
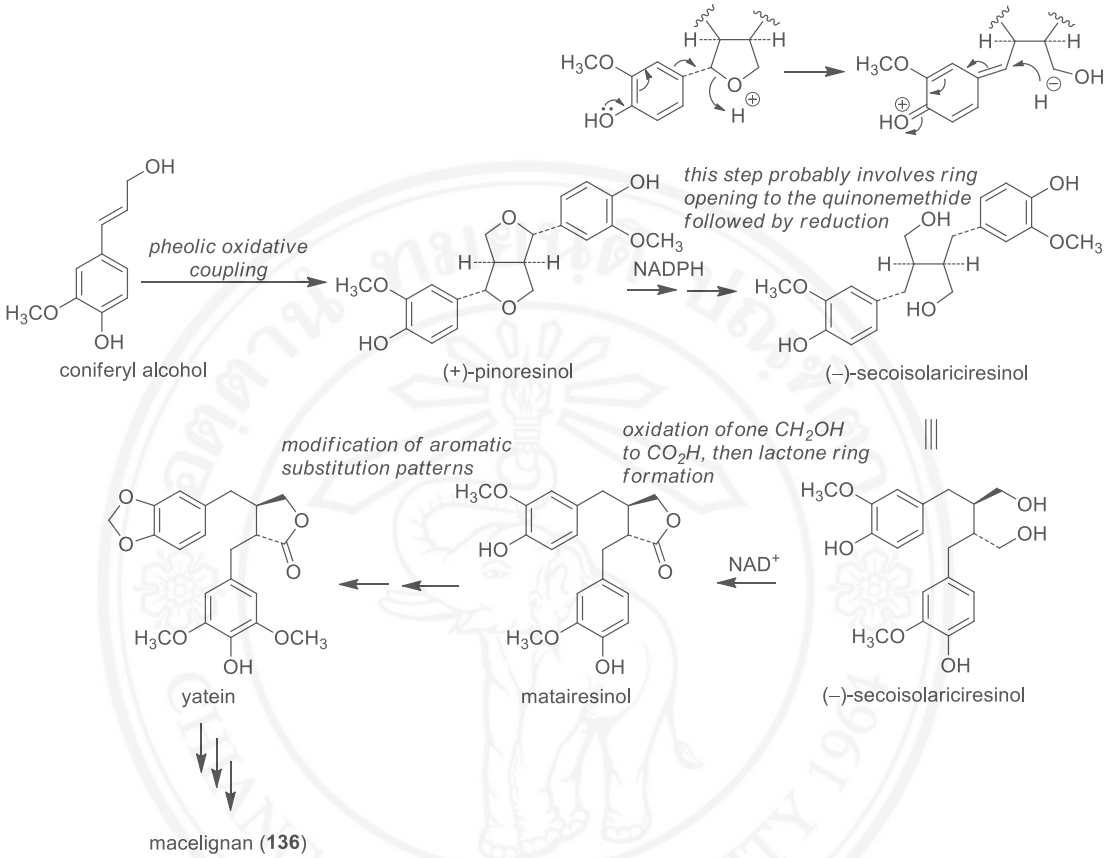


Figure 3.16 Mechanism of key fragment ions of macelignan (**136**) from EIMS

Biosynthesis of macelignan (**136**) that lignan was occurred as the same eugenol *via* coniferyl alcohol in shikimate pathway as shown in Scheme 3.6 [66].



Scheme 3.3 Biosynthesis of macelignan (**136**) *via* Shikimate pathway

The mixture of phytosterol; *β*-sitosterol (**137**) and stigmasterol (**70**) were obtained as colorless solids with a melting point in range of 138.7–144.8 °C compared with mp 144–146 °C from the previous study [67]. EIMS spectrometry showed molecular ion peaks at m/z 414 and 412 $[M]^+$ that correspond to the molecular formula of $C_{29}H_{50}O$ and $C_{29}H_{48}O$, respectively. Ion peaks were also observed at *m/z* 225, 159, 133 and 55. Other ion peaks were further observed at *m/z* 329, 273, 303 and 145. On subjection to IR spectroscopic analysis, the observed absorption band at 3396 cm^{-1} is characteristic of O-H stretching. Absorption at 1642 cm^{-1} is due to C=C of alkene groups. The ^1H NMR spectrum revealed the existence of signals for olefinic proton at δ 5.43 (1H, *m*, H-6) and oxygenated proton at δ 3.52 (1H, *m*, H-3). From ^1H NMR spectral data (Figure 3.18), it was found that the spectrum show mixture of compounds **137** and **70** in the

approximately ratio 1:1, when comparison with *β*-sitosterol (Aldrich S340-2) and stigmasterol (Aldrich S440-9) [68] as shown in Figure 3.19. In olefinic proton show signal at *δ* 5.43 (1H, *m*, H-6), has two proton in integration 1.01 (1H of *β*-sitosterol and 1H of stigmasterol). In addition, the position of olefinic proton at *δ* 5.14 (1H, *dd*, *J* = 15.2, 8.6 Hz, H-22) and 5.01 (1H, *dd*, *J* = 15.2, 8.6 Hz, H-23) show integration 0.43 and 0.43, which is only stigmasterol. So that, this spectrum indicated two mixed compounds as shown in Table 3.10. The spectroscopic data let to the identification of *β*-sitosterol and stigmasterol, which confirmed by comparison with the literature data [67, 69, 70].

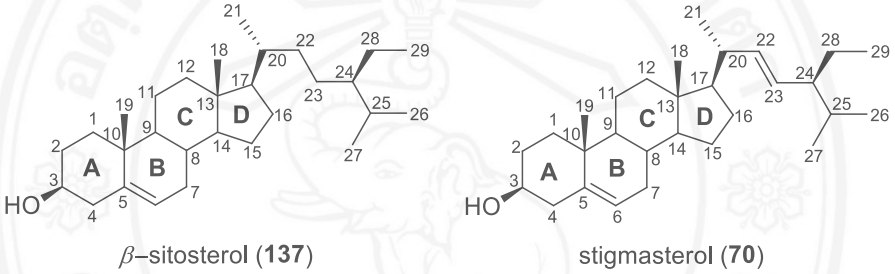


Figure 3.17 Labeling number of each carbon in structure of *β*-sitosterol (**137**) and stigmasterol (**70**)

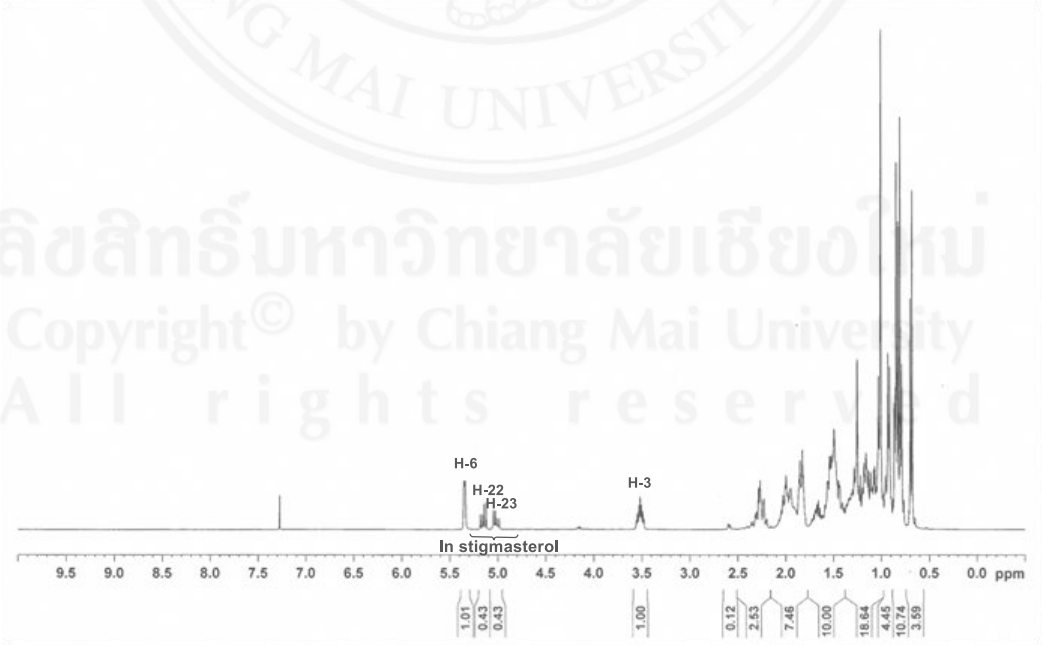
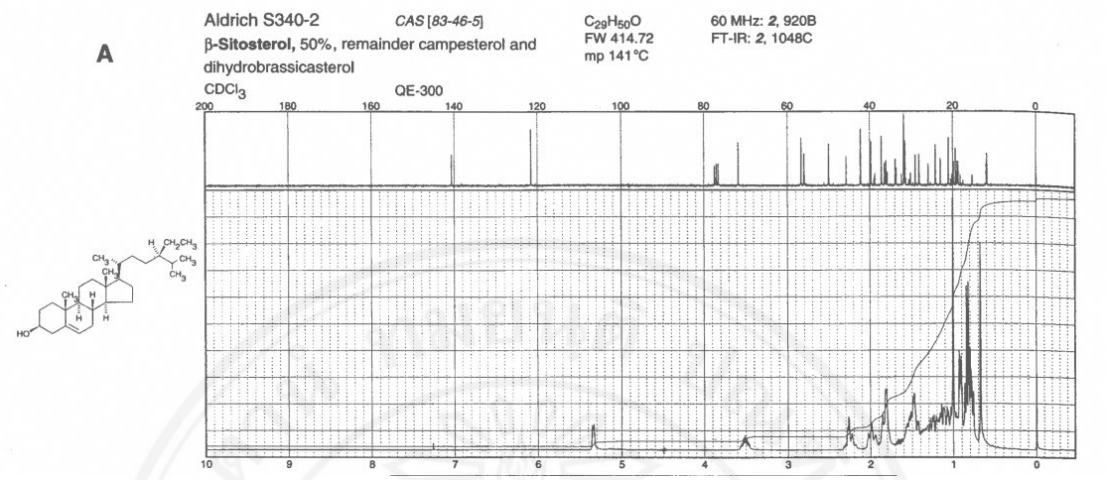
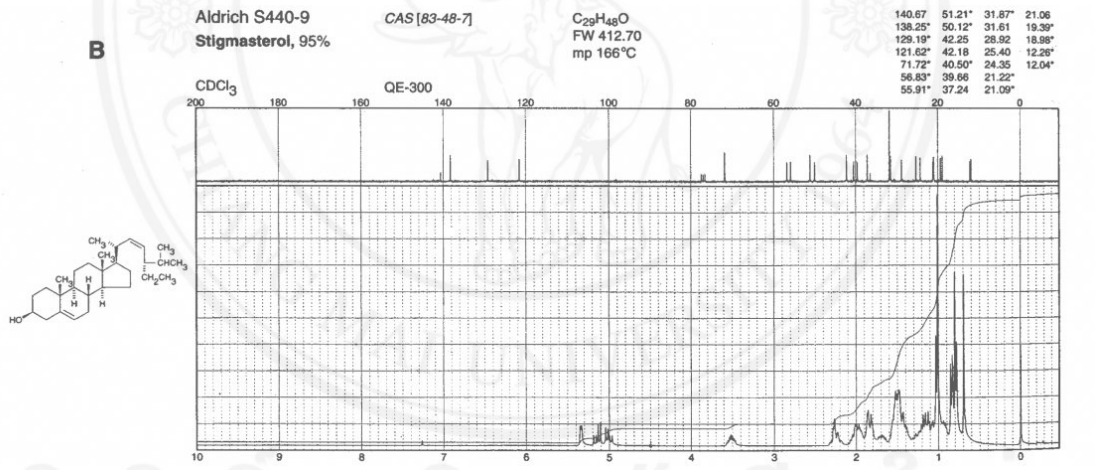


Figure 3.18 ^{1}H NMR spectra data of *β*-sitosterol (**137**) and stigmasterol (**70**)



(a)



(b)

Figure 3.19 ^{1}H NMR spectra data of references of steroids

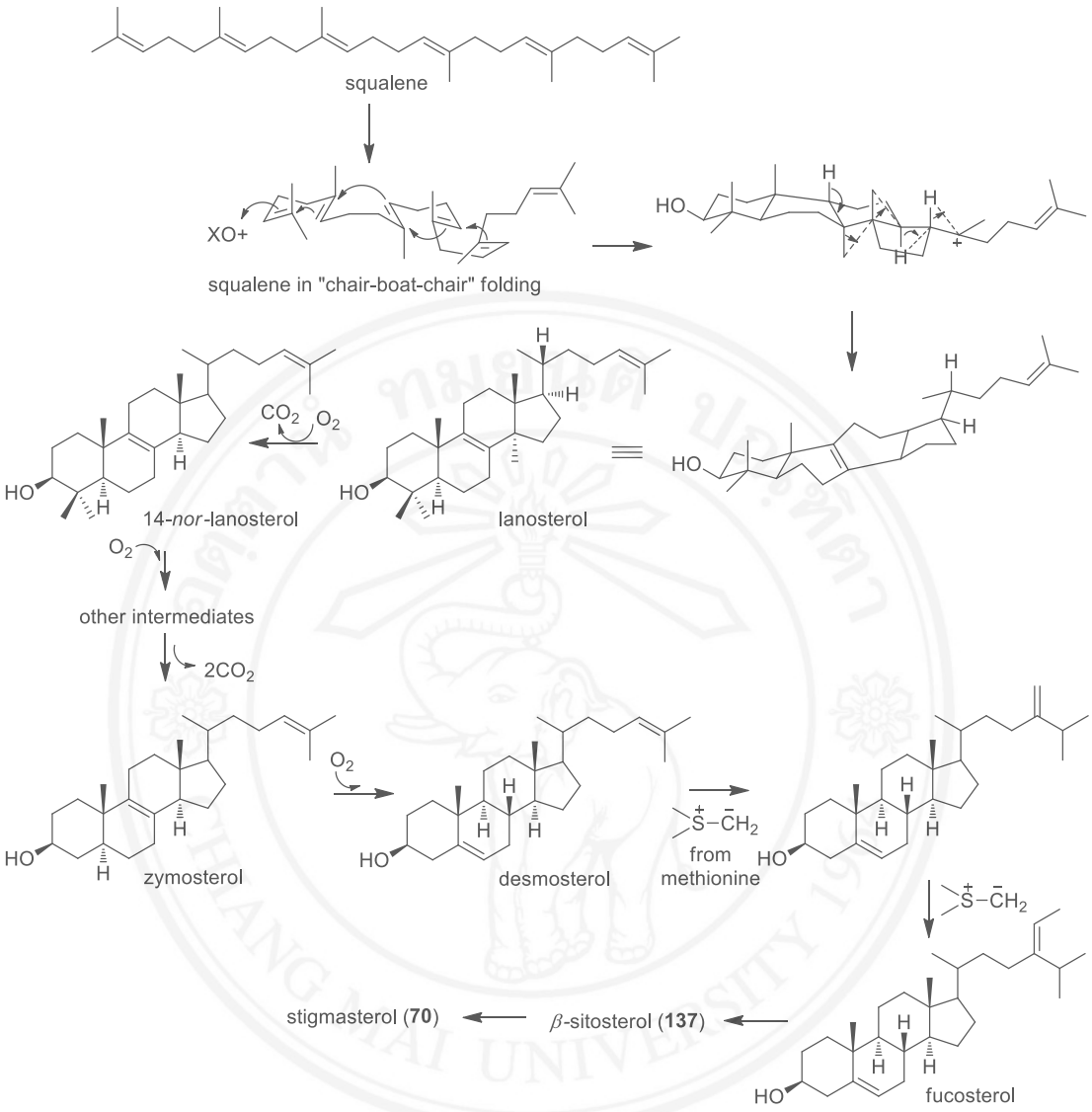
a) *β*-sitosterol and b) stigmasterol

Table 3.10 ^{1}H NMR data of *β*-sitosterol (**137**) and stigmasterol (**70**)

Positions	*β*-sitosterol (Mult., *J* in Hz)		stigmasterol (Mult., *J* in Hz)	
	δ_H	δ_H [67]	δ_H	δ_H [69]
3	3.52 (*m*)	3.52 (*m*)	3.52 (*m*)	3.53 (*m*)
6	5.43 (*m*)	5.358 (*br s*)	5.43 (*m*)	5.36 (*t*)
18	0.69 (*s*)	0.68 (*s*)	0.69 (*s*)	0.68
19	1.02 (*s*)	1.01 (*s*)	1.02 (*s*)	1.02
21	1.00 (*s*)	0.92 (*d*, 6.4)	1.00 (*s*)	0.92
22	-	-	5.14 (*dd*, 15.2, 8.6)	5.15 (*s*)
23	-	-	5.01 (*dd*, 15.2, 8.6)	5.01 (*s*)
26	0.84 (*d*, 6.5)	0.814 (*d*, 6.5)	0.84 (*d*, 6.5)	0.86
27	0.79 (*d*, 7.0)	0.833 (*d*, 6.5)	0.79 (*d*, 7.0)	0.79
29	0.81 (*t*)	0.845 (*t*, 7.5)	0.81 (*t*)	0.82

Recorded in $CDCl_3$

Two steroids, *β*-sitosterol (**137**) and stigmasterol (**70**) consist of six isoprene units. They derived from five-carbon of isoprenoids, isopentyl diphosphate (IPP) and diphosphate (DMAPP) to the formation of the C-30 and originated exclusively from the Mevalonate pathway. They are mainly made up of squalene, via the triterpene lanosterol, into desmosterol and fucosterol [57, 71] as shown in Scheme 3.4.



Scheme 3.4 Biosynthesis of *β*-sitosterol (**137**) and stigmasterol (**70**) *via* Mevalonate pathway

3.2 Structural elucidation of pure compounds in *Cleistocalyx nervosum* var. *paniala* seeds

The partially purified fractions (MK1–6), from isolated crude CH_2Cl_2 extract of *C. nervosum* var. *paniala* seeds by using silica gel chromatography, were evaluated their mutagenicity using Salmonella mutation assay in TA100 with or without metabolic activation (S9 mix) by Wanida Inboot' s Thesis [72] as shown in Table 3.11

Table 3.11 Mutagenicity of partially purified fractions isolated CH_2Cl_2 extract of *C. nervosum* var. *paniala* seeds using Salmonella mutation assay in TA100 with or without metabolic activation (S9 mix)

Treatment	Average of His^+ revertant colonies (MI)			
	–S9		+S9	
AF-2 0.01 μg/pl	718 ± 58.1	(7.1)	N.A.	-
2-AA 0.5 μg/pl	N.A.	-	883 ± 15.7	(7.4)
DMSO 50 μg/pl	101 ± 16.9	(1.0)	119 ± 21.7	(1.0)
MK1 10 μg/pl	110 ± 18.6	(1.1)	123 ± 18.6	(1.0)
MK1 100 μg/pl	98 ± 16.8	(1.0)	132 ± 21.9	(1.1)
MK2 10 μg/pl	89 ± 15.6	(0.9)	129 ± 27.8	(1.1)
MK2 100 μg/pl	68 ± 10.4	(0.7)	102 ± 13.3	(0.9)
MK3 10 μg/pl	74 ± 16.3	(0.7)	122 ± 28.5	(1.0)
MK3 100 μg/pl	73 ± 12.3	(0.7)	100 ± 16.8	(0.8)
MK4 10 μg/pl	57 ± 11.4	(0.6)	103 ± 17.7	(0.9)
MK4 100 μg/pl	14 ± 3.5 (K)	(0.1)	46 ± 6.0 (K)	(0.4)
MK5 10 μg/pl	107 ± 12.7	(1.1)	115 ± 17.5	(1.0)
MK5 100 μg/pl	117 ± 14.2	(1.2)	201 ± 82.4	(1.7)
MK6 10 μg/pl	96 ± 19.3	(1.0)	141 ± 27.5	(1.2)
MK6 100 μg/pl	116 ± 21.2	(1.2)	130 ± 26.4	(1.1)

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, 2-AA: 2-aminoanthracene, DMSO; dimethyl sulfoxide NA: not analyzed, K: killing effect, MI: mutagenicity index, The results showed number of revertant colonies of bacteria as a Mean ± SEM

From the result of mutagenicity using Salmonella mutation assay in TA100 with or without metabolic activation (S9 mix). It was found that, Fraction MK4 has high potential more than other fractions and fraction MK3 has minor effect from MK4. Therefore, we are interested to separate in fractions MK3 and 4.

Phytochemical studies of fractions MK3 and 4 in the seeds of *C. nervosum* var. *paniala* afforded two known compounds namely 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC (**128**) and hariganetin (**138**) in minor and major, respectively.

Two compounds isolated from the seeds of *C. nervosum* var. *paniala*, were test using Salmonella mutation assay against AFB1, MeIQ and AF-2. The results of bioactivity tests from *Department of Biochemistry, Faculty of Medicine, Chiang Mai University* [72], were shown in Table 3.12.

Table 3.12 % Inhibition of DMC (**128**) and hariganetin (**138**) using Salmonella mutation assay against AFB1, MeIQ and AF-2 induced mutagenesis

Treatment	% Inhibition		
	AFB1 (5 ng/pl)	MeIQ (12.5 ng/pl)	AF-2 (10 ng/pl)
DMC 2 μg/pl	98.6 ± 6.3	83.6 ± 4.3	NA
DMC 10 μg/pl	100.0 ± 5.4	99.5 ± 0.3	56.8 ± 22.0
DMC 50 μg/pl	NA	NA	70.9 ± 14.1
Hariganetin 2 μg/pl	11.5 ± 8.4	49.3 ± 10.7	NA
Hariganetin 10 μg/pl	63.5 ± 6.1	58.4 ± 5.5	40.0 ± 29.4
Hariganetin 50 μg/pl	NA	NA	56.0 ± 12.3

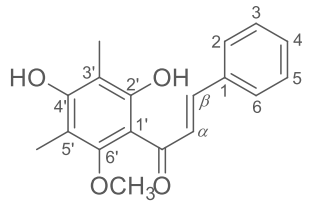
AFB1: aflatoxin B1, MeIQ: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, NA: not analyzed, % inhibition showed as a Mean ± SD

The structures of all isolated compounds were identified by interpretation of their spectral data of IR, EIMS, ^{1}H and ^{13}C NMR spectra including DEPT, COSY, HMQC and HMBC experiments, as well as by comparison of their spectral data with those reported in the literature.

2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC (**128**); had been isolated from fractions MK3 and MK4 of the CH_2Cl_2 extract of *C. nervosum* var. *paniala* seeds. This compound was an orange solids with a melting point 120.8–122.3 °C compared with mp 125–126 °C from the previous study [73] and analyzed for $C_{18}H_{18}O_4Na$ by means of HRMS measurement on the $[M+Na]^+$ ion *m/z* 321.1102 calcd for 321.1103. The EIMS exhibited a molecular ion peak at *m/z* 298 $[M^+]$, corresponding to a molecular formula of $C_{18}H_{18}O_4$ and fragmentation ions were found signals at *m/z* 221, 194, 166, 103 and 77 compared with the literature [74]. The IR spectrum of this compound showed a characteristic C=O stretching band of the conjugated carbonyl system at 1628 cm^{-1}. The absorption band at 3444 cm^{-1} was assigned to a free hydroxyl as well as a H-bonded hydroxyl group. Its ^1H NMR (400 MHz) spectrum of compound **128** exhibited signals for monosubstituted phenyl group at δ 7.41 and 7.64, a *trans*-disubstituted double bond at δ 7.84 and 7.99 (J_{AB} = 16.0 Hz) and two benzylic methyl groups at δ 2.14 and 2.16 ppm. Furthermore, the spectrum revealed the presence of three singlets for dihydroxyl and methoxyl group at δ 13.69 (2′-OH), 5.38 (4′-OH) and 3.66 (6′-OCH_3) ppm, respectively. Thus, from the above spectral data, it became clear that the chalcone had a 2',4',6'-trioxygenation pattern which could lead to the structure 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone.

The ^{13}C NMR (100 MHz) of compound **128** in $CDCl_3$ showed the presence of carbon carrying one methoxyl and two hydroxyl groups with signals at δ 158.8, 162.0 and 159.3, respectively, along with low field signal at δ 193.4 due to a chelated carbonyl carbon. The signals from DEPT-135 and DEPT-90 spectra, displayed 18 carbons as shown below;

$3 \times CH_3$	at δ	7.6, 8.2, 62.3
$7 \times CH$	at δ	126.7, 128.4, 128.4, 128.9, 128.9, 130.2, 135.3
$8 \times C$	at δ	106.6, 109.0, 109.0, 135.3, 158.8, 159.3, 162.0, 193.4.



DMC (**128**)

Figure 3.20 Labeling number of each carbon in structure of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC (**128**)

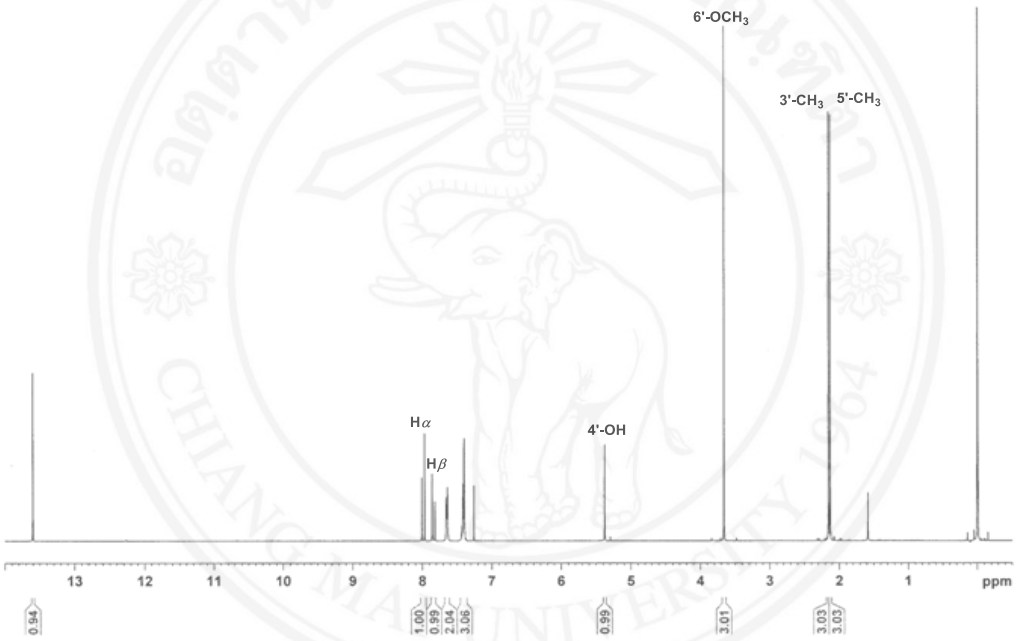


Figure 3.21 ^{1}H NMR spectrum of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

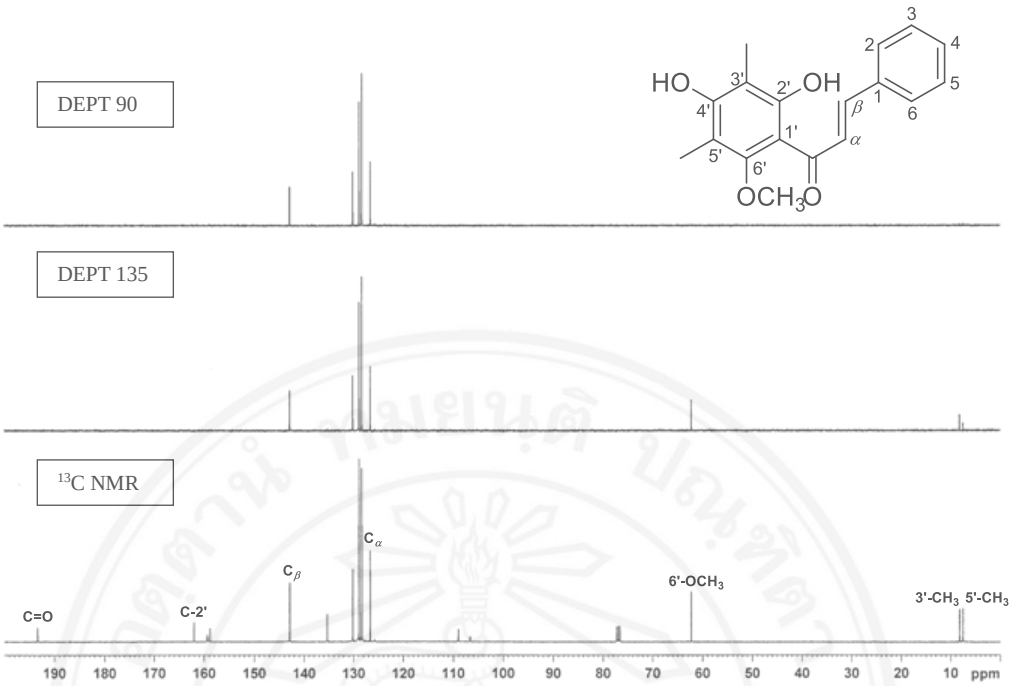


Figure 3.22 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

The complete structure of **128** was determined by analyzing the 2D-NMR data including the HMQC and HMBC spectra in $CDCl_3$. The HMQC spectrum allowed us to connect the protons and carbons as shown in Table 3.13. The HMBC spectrum showed correlation of proton signal at δ 7.84 (*d*, *J* = 15.7 Hz, H_β) with the carbon signals at δ 135.3 (C-1), 128.9 (C-2,6), 126.7 (C_α) and 193.4 (C=O). The proton signal at δ 7.99 (*d*, *J* = 15.7 Hz, H_α) correlated with the carbon signals at δ 135.3 (C-1), 142.9 (C_β), 193.4 (C=O) and 106.6 (C-1′). Proton signals of dimethyl group at δ 2.14 (*s*, 3-C$\underline{H}_3$) showed correlation with the carbon signal at δ 162.0 (C-2′), 109.0 (C-3′) and 159.3 (C-4′) and signals at δ 2.16 (*s*, 3-C$\underline{H}_3$) showed correlation with the carbon signal at δ 159.3 (C-4′), 109.0 (C-5′) and 158.8 (C-6′) and proton signal of methoxy group at 3.66 (*s*, 6-OC$\underline{H}_3$) correlated with the carbon signal at δ 158.8 (C-6′). These results were the same as reported in the literature [73–76]. We assigned the structure of compound **128** as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone.

Table 3.13 ^{1}H and ^{13}C NMR data of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [74] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [74] (ppm)
1	-	-	135.3	135.5
2	7.64 (*m*)	7.63 (*m*)	128.9	128.9
3	7.41 (*m*)	7.41 (*m*)	128.4	128.4
4			130.2	130.2
5			128.4	128.4
6	7.64 (*m*)	7.63 (*m*)	128.9	128.9
β	7.84 (*d*, 15.7)	7.84 (*d*, 15.7)	142.9	142.9
α	7.99 (*d*, 15.7)	8.00 (*d*, 15.7)	126.7	126.7
C=O	-	-	193.4	193.4
1′	-	-	106.6	106.6
2′	-	-	162.0	162.0
3′	-	-	109.0	109.0
4′	-	-	159.3	159.3
5′	-	-	109.0	109.0
6′	-	-	158.8	158.8
2′-OH	13.69 (*s*)	13.69 (*s*)	-	-
3′-CH_3	2.14 (*s*)	2.15 (*s*)	8.2	8.2
4′-OH	5.38 (*s*)	5.88 (*s*)	-	-
5′-CH_3	2.16 (*s*)	2.17 (*s*)	7.6	7.6
6′-OCH_3	3.66 (*s*)	3.66 (*s*)	62.3	62.3

Recorded in $CDCl_3$

Table 3.14 ^{1}H-^{1}H COSY, HMQC, HMBC data of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**) ($CDCl_3$) at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^{1}H-^{1}H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
2	H-3	C-2	C-3, 4, β
3	H-2, 4	C-3	C-1, 2, 5
4	H-3, 5	C-4	C-2, 3, 5, 6
5	H-4, 6	C-5	C-1, 3, 6
6	H-5	C-6	C-2, 4, 5
β	H_α	C_β	C-1, 2, 6, α, -CO-
α	H_β	C_α	C-1, β, -CO-
3′-CH_3	-	3′-CH_3	C-2′, 3′, 4′
5′-CH_3	-	5′-CH_3	C-4′, 5′, 6′
6′-OCH_3	-	6′-OCH_3	C-6′

Recorded in $CDCl_3$

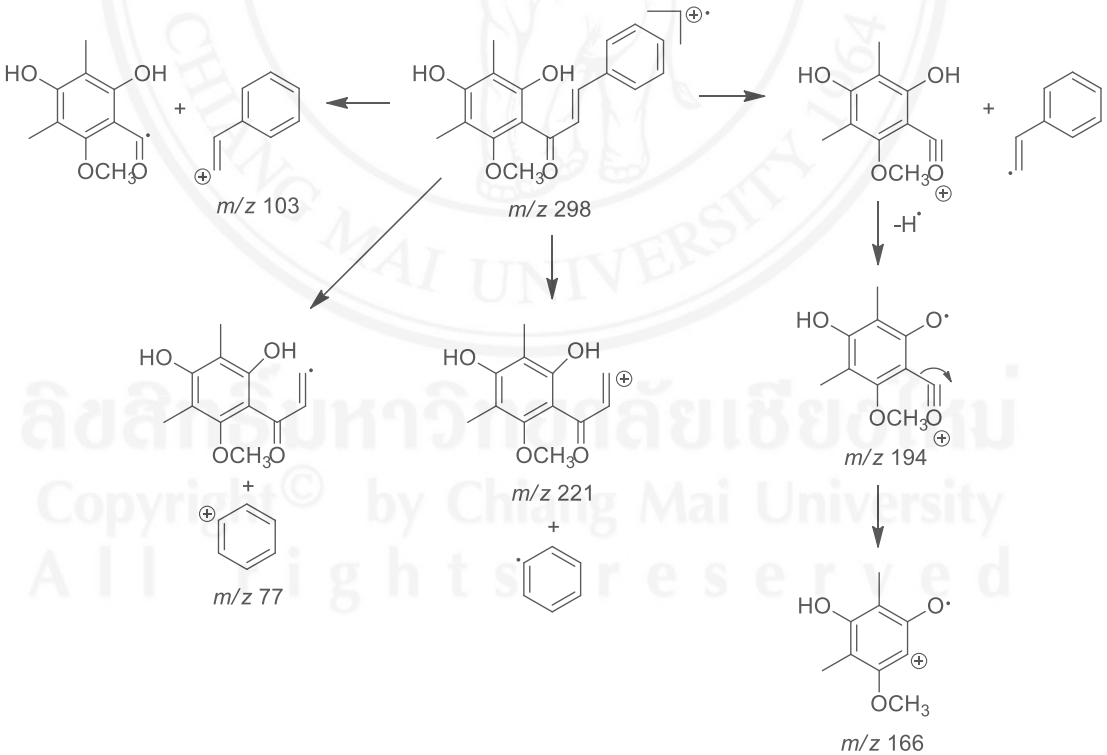
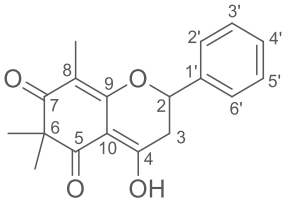


Figure 3.23 Mechanism of key fragment ions of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**) from EIMS

Hariganetin (**138**); 4-hydroxy-6,6,8-trimethyl-2-phenyl-2*H*-1-benzopyran-5,7(3*H*, 6*H*)-dione for a systematic name, was isolated as orange solids by crystallization from EtOAc/hexane with a melting point 140.4–141.5 °C. It was determined as $C_{18}H_{18}O_4Na$ by HRMS which showed the $[M+Na]^+$ ion *m/z* 321.1102 calcd for 321.1103. EIMS exhibited a molecular ion peak at *m/z* 298 $[M^+]$ (Figure 3.27), corresponding to a molecular formula of $C_{18}H_{18}O_4$. The IR spectrum of **138** exhibited the C=O stretching of two carbonyl group at 1619 and 1649 cm^{-1} and the C=C stretching of conjugated carbonyl was also observed at 1449 cm^{-1}. The bands at 3450 and 1168 cm^{-1} were referred to the O-H and C-O stretching, respectively.

The ^1H NMR spectrum of compound **138** (Figure 3.25) in $CDCl_3$ showed trimethyl protons appeared singlets at δ 1.40, 1.42 and 1.86 ppm (9H, *s*, 6,6,8-C<u>H</u>$_3$ respectively) and one hydroxyl proton at δ 15.80 (*s*, 4-O<u>H</u>) for hydrogen bonding of carbonyl group were observed. The proton at 3-position appeared a doublet of doublet at δ 2.92 (J = 17.9, 3.7 Hz) and 3.03 (J = 17.9, 10.8 Hz) and oxymethine proton H-2 showed doublet of doublets signal at δ 5.32 (J = 10.8, 3.7 Hz) ppm. The ^{13}C NMR spectrum of this compound displayed several characteristic signals, C-4 carbon that had hydroxyl group had signal at δ 182.8 ppm, two carbonyl carbon C-5 and C-7 showed peaks at δ 198.0 and 201.7 ppm, respectively and C-9 carbon of endocyclic double bond showed signal at δ 161.2 ppm (Figure 3.26 and Table 3.15). In combination with DEPT-135 and DEPT-90 spectra, showed 15 signals led to the possible classification of the 18 carbons as shown below.

$3 \times CH_3$	at δ	7.9, 23.1, 25.5
$1 \times CH_2$	at δ	38.2
$6 \times CH$	at δ	76.0, 125.8, 125.8, 128.9, 128.9, 128.9
$8 \times C$	at δ	52.3, 101.4, 107.3, 138.0, 161.2, 182.8, 198.0, 201.7



hariganetin (**138**)

Figure 3.24 Labeling number of each carbon in structure of hariganetin (**138**)

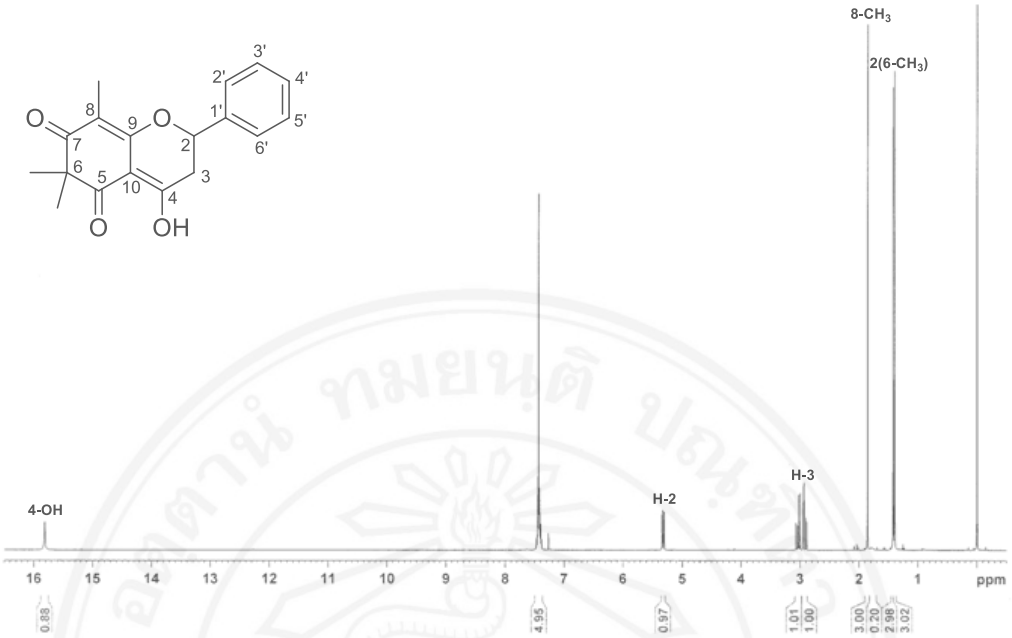


Figure 3.25 ^{1}H NMR spectrum of hariganetin (**138**)

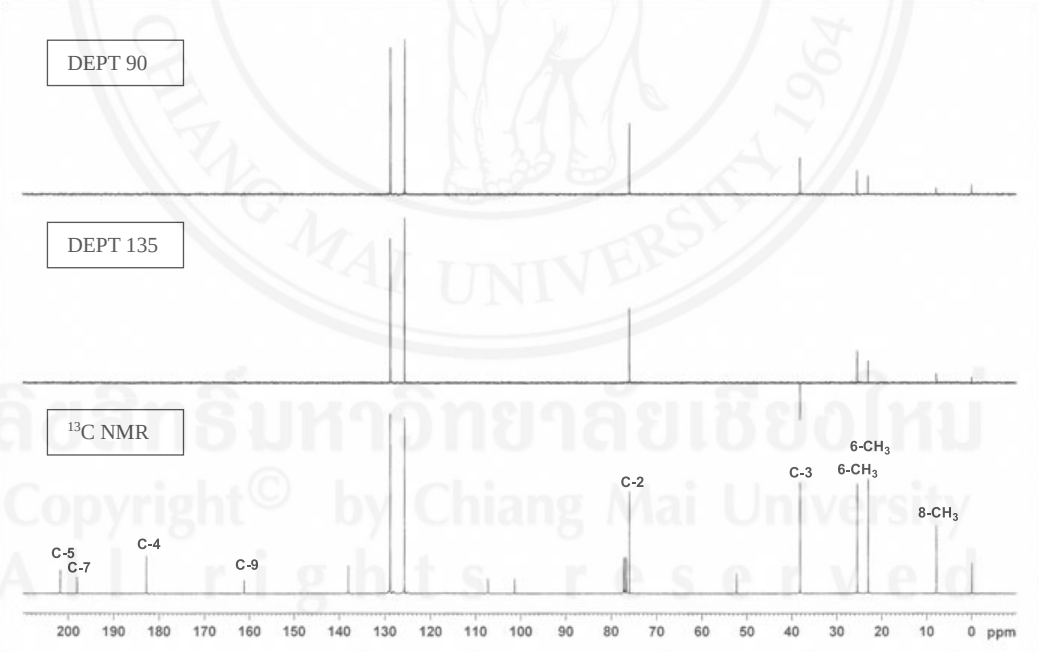
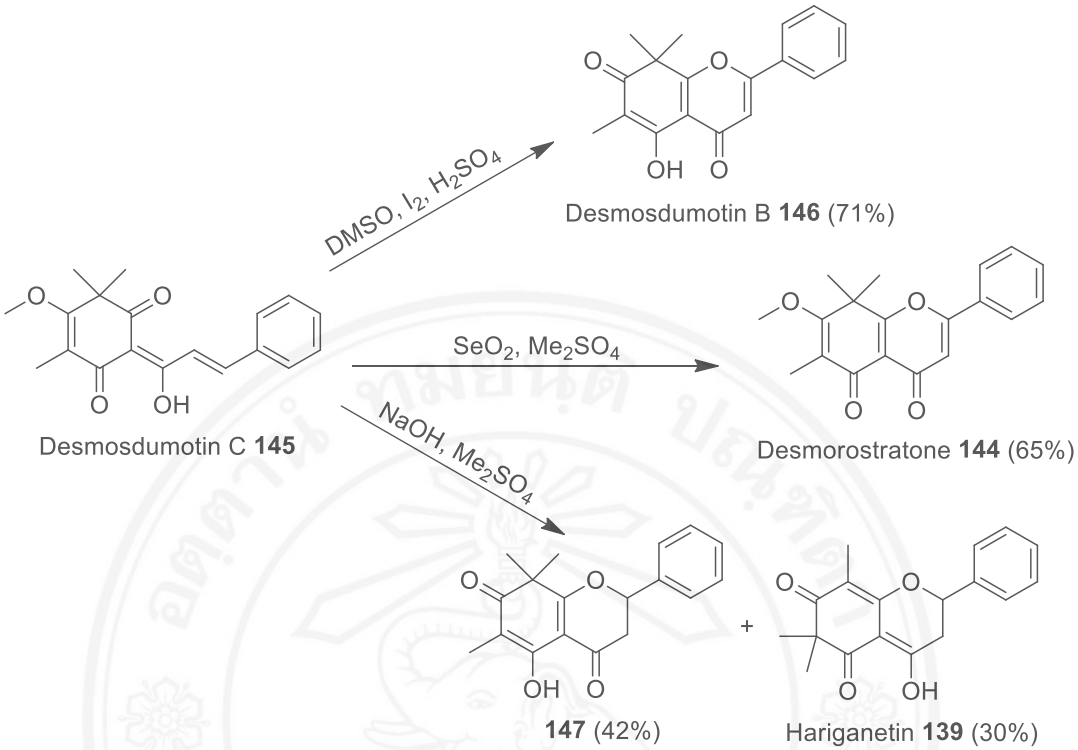


Figure 3.26 DEPT 90, DEPT 135 and ^{13}C NMR spectra of hariganetin (**138**)

Furthermore, the 2D-NMR spectra, HMQC and HMBC were also recorded for this compound to confirm the assignment as shown in Table 3.16. The HMQC data was used to explain correlation between ^{1}H and ^{13}C NMR spectra of this compound. In the result, it displayed the correlation such as H-2 proton was coupled by C-2 carbon, H-3 proton associated with C-3 carbon, and trimethyl protons showed correlation with 8-CH_3 and 6-$(CH_3)_2$ carbons at 7.9, 23.1, and 25.5 ppm, respectively.

The HMBC spectrum showed correlation of proton signal at δ 2.92 (*dd*, *J* = 17.9, 3.7 Hz) and 3.03 (*dd*, *J* = 17.9, 10.8 Hz) of methylene proton H-3 with the carbon signals at δ 76.0 (C-2), 182.8 (C-4), 101.4 (C-10), and 138.0 (C-1′), the proton signal at δ 5.32 (*dd*, *J* = 10.8, 3.7 Hz, H-2) related with the carbon signals at δ 38.2 (C-3), 182.8 (C-4), 138.0 (C-1′), and 125.8 (C-2′,6′), the proton signal at δ 1.86 (*s*, 8-C$\underline{H}_3$) showed association with the carbon signal at δ 198.0 (C-7), 107.3 (C-8) and 161.2 (C-9) and proton signals of dimethyl group at δ 1.40 and 1.42 (*s*, 6- C$\underline{H}_3$) showed correlation with the carbon signal at δ 201.7 (C-5), 52.3 (C-6) and 198.0 (C-7).

Additionally, the spectroscopic data of compound **138** was found to consistent with some structural characteristic in the molecules of flavonoid, reported by Ngoc *et al.*, 2009 [77] and separated from *Myrica gale* seeds [78]. Therefore, the structure of compound **138** was deduced as hariganetin, which has been synthesized desmorostratone (**144**) from desmosdumotin C (**145)** (Scheme 3.5). However, hariganetin (**138**) was report for the first time from genus *Cleistocalyx*.



Scheme 3.5 Synthesis of desmorostratone (**144**)

Table 3.15 ^{1}H and ^{13}C NMR data of hariganetin (**138**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [77] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [77] (ppm)
2	5.32 (*dd*, 10.8, 3.7)	5.32 (*dd*, 11.0, 3.5)	76.0	76.1
3	2.92 (*dd*, 17.9, 3.7)	2.92 (*dd*, 18.0, 3.5)	38.2	38.3
	3.03(*dd*, 17.9, 10.8)	3.03(*dd*, 18.0, 11.0)		
4	-	-	182.8	182.9
5	-	-	201.7	201.8
6	-	-	52.3	52.4
7	-	-	198.0	198.1
8	-	-	107.3	107.4
9	-	-	161.2	161.3
10	-	-	101.4	101.5
1′	-	-	138.0	138.1

Table 3.14 (Continued)

Positions	δ_H (Mult., *J* in Hz)	δ_H [77] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [77] (ppm)
2′	7.37–7.48 (*m*)	7.39–7.47 (*m*)	125.8	125.8
3′			128.9	129.0
4′			128.9	128.9
5′			128.9	129.0
6′			125.8	125.8
4-OH	15.80(*s*)	15.80(*s*)		-
6-CH_3	1.40 (*s*)	1.40 (*s*)	23.1	23.1
6-CH_3	1.42 (*s*)	1.42 (*s*)	25.5	25.5
8-CH_3	1.86 (*s*)	1.86 (*s*)	7.9	7.9

Recorded in $CDCl_3$

Table 3.16 ^{1}H-^{1}H COSY, HMQC, HMBC data of hariganetin (**138**) ($CDCl_3$) at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR)

Proton Position	^{1}H-^{1}H COSY (Coupling of H)	HMQC (Correlation of C)	HMBC (Correlation of C)
2	H-3	C-2	C-3, 4, 1′, 2′, 6′
3	H-2	C-3	C-2, 4, 10, 1′
2′	H-3′	C-2′	C-2, 1′, 3′, 4′, 6′
3′	H-2′, 4′	C-3′	C-1′, 2′, 4′, 5′
4′	H-3′, 5′	C-4′	C-2′, 3′, 5′, 6′
5′	H-4′, 6′	C-5′	C-1′, 3′, 4′, 6′
6-C<u>H</u>$_3$	-	6-C<u>H</u>$_3$	C-5, 6, 7
6-C<u>H</u>$_3$	-	6-C<u>H</u>$_3$	C-5, 6, 7
8-C<u>H</u>$_3$	-	8-C<u>H</u>$_3$	C-7, 8, 9

Recorded in $CDCl_3$

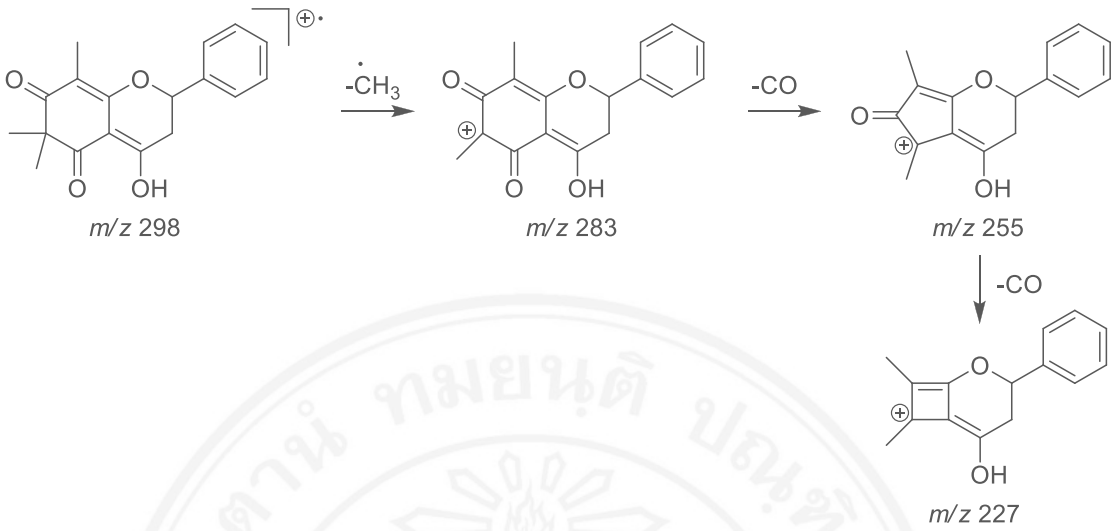
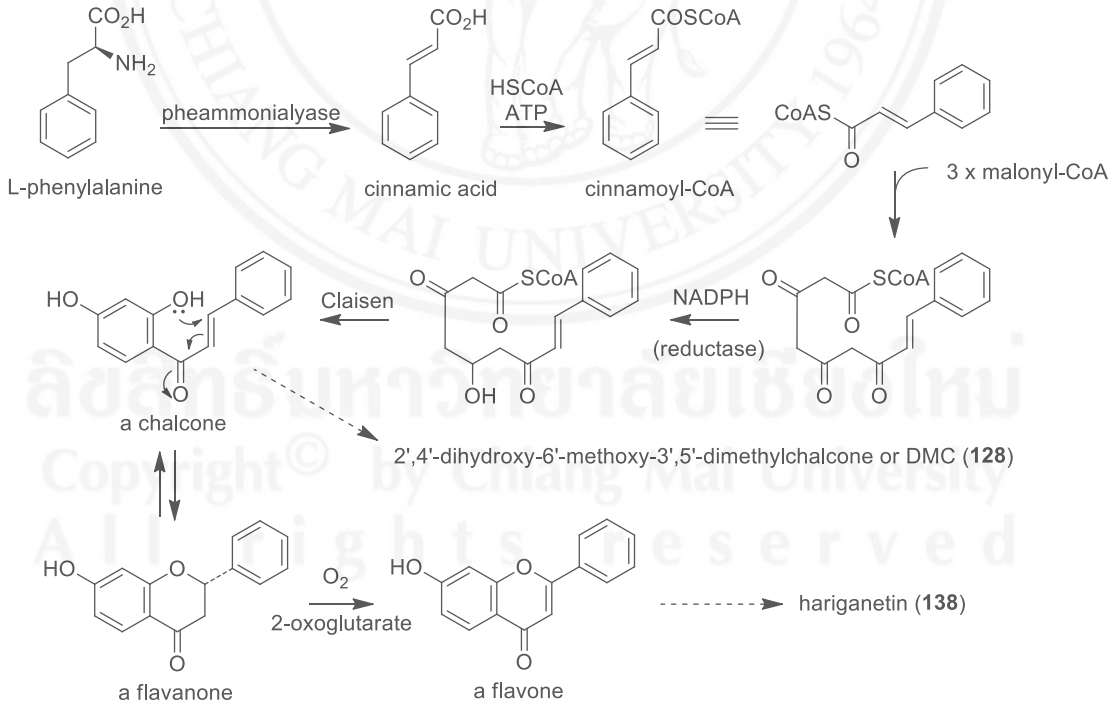


Figure 3.27 Mechanism of key fragment ions of hariganetin (**138**) from EIMS

2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC **(128),** as a chalcone, and hariganetin (**138**), as a flavones, were well known compounds which had biosynthesis from Shikimate pathway *via* the precursor of phenylalanine as shown in Scheme 3.8 [57, 79].



Scheme 3.6 Biosynthesis of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC (**128**) and hariganetin (**138**) *via* Shikimate pathway

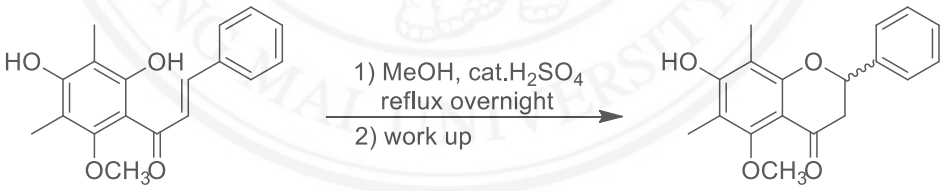
3.3 Synthetic derivatives of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) (128)

The result of bioactivity tests using Salmonella mutation assay against AFB1, MeIQ and AF-2 induced mutagenesis was shown in Table 3.16. It was found that, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone or DMC has high potential antimutagenic compound more than hariganetin. Therefore, we will modify the structure of DMC and investigate their bioactivities. Structure modification of DMC achieved by oxidation, reduction and methylation reaction as shown in Table 3.17.

Table 3.17 Conditions for reaction from structure modification of DMC

Entry	Conditions	Result
1	1.5 eq. H_2O_2, 6 N NaOH, 15–20°C	Complex mixture
2	1.5 eq. *m*-CPBA, anhyd.$CHCl_3$, reflux overnight	Recover of starting material
3	MeOH, H_2SO_4 cat., reflux overnight	See 2.4.1 and 3.3.1
4	3.2 eq. $NaBH_4$, THF:MeOH (1:1), 0°C	See 2.4.2 and 3.3.2
5	10 eq. K_2CO_3, CH_3I, anhyd.acetone, rt	See 2.4.3 and 3.3.3

3.3.1 7-Hydroxy-5-methoxy-6,8-dimethylflavanone (139)



128 → **139**, 70% yield

The refluxing of DMC (**128**) in MeOH with a catalytic amount of conc. H_2SO_4, and purification by flash column chromatography (silica gel) using EtOAc:hexane (3:7) as eluent afforded the racemic *7-hydroxy-5-methoxy-6,8-dimethylflavanone* (**139**) in 70% yield and 92% conversion from the starting material. It was found that a white solids with a melting point 210.0–211.2 °C compared with mp 210–211°C from the previous study [76] and exhibited a molecular ion peak in ESIMS at *m/z* 321.1099 $[M+Na]^+$ (calculation 321.1103, positive ion mode), corresponding to a molecular formula of $C_{18}H_{18}O_4Na$. The IR spectrum showed absorption band of

hydroxyl group at 3395 cm^{-1}, conjugated carbonyl group at 1650 cm^{-1} and ether group at 1196 cm^{-1}. The ^1H NMR spectral data also justified our assignment of a flavanone skeleton for compound **139** with comparison the literature [76, 80]. In particular, the one proton doublet of doublets at δ 5.41 (J = 13.0, 3.0 Hz) for H-2 is distinctive for a flavanone, as is the splitting pattern observed for the C-3 methylene (δ 45.7) which produces a set of doublet of doublets at δ 2.97 for the C-3 axial proton (J = 16.7, 13.0 Hz), and another set of doublet of doublets at δ 2.83 for the C-3 equatorial proton (1H, *dd*, J = 16.7, 3.1 Hz) (Figure 3.28 and Table 3.18).

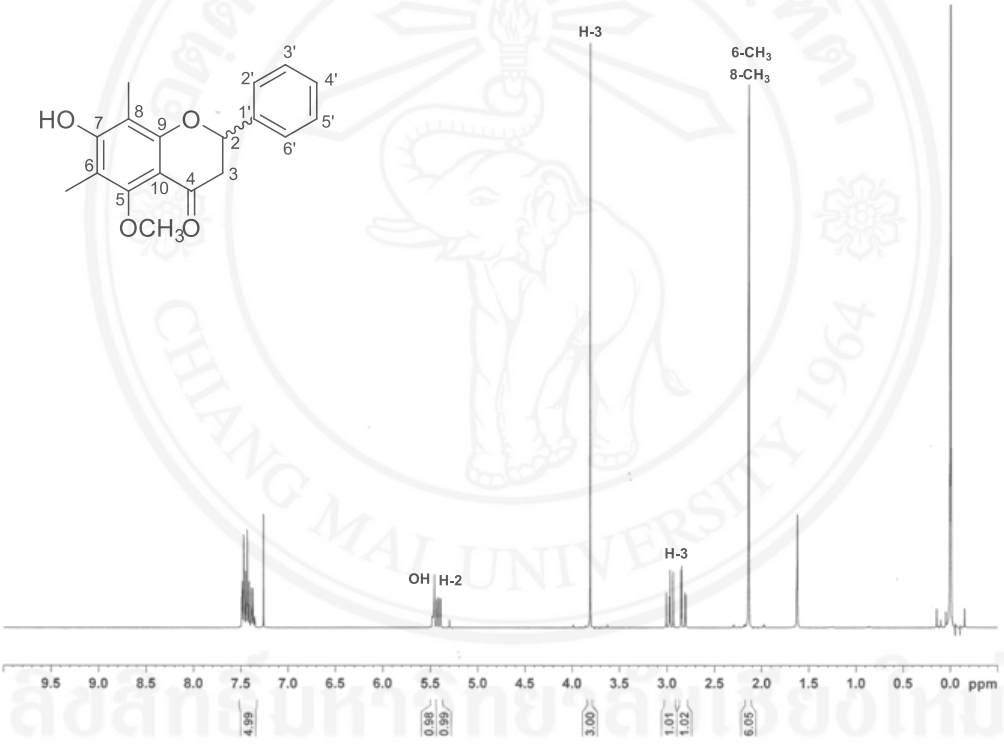


Figure 3.28 ^1H NMR spectrum of 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**139**)

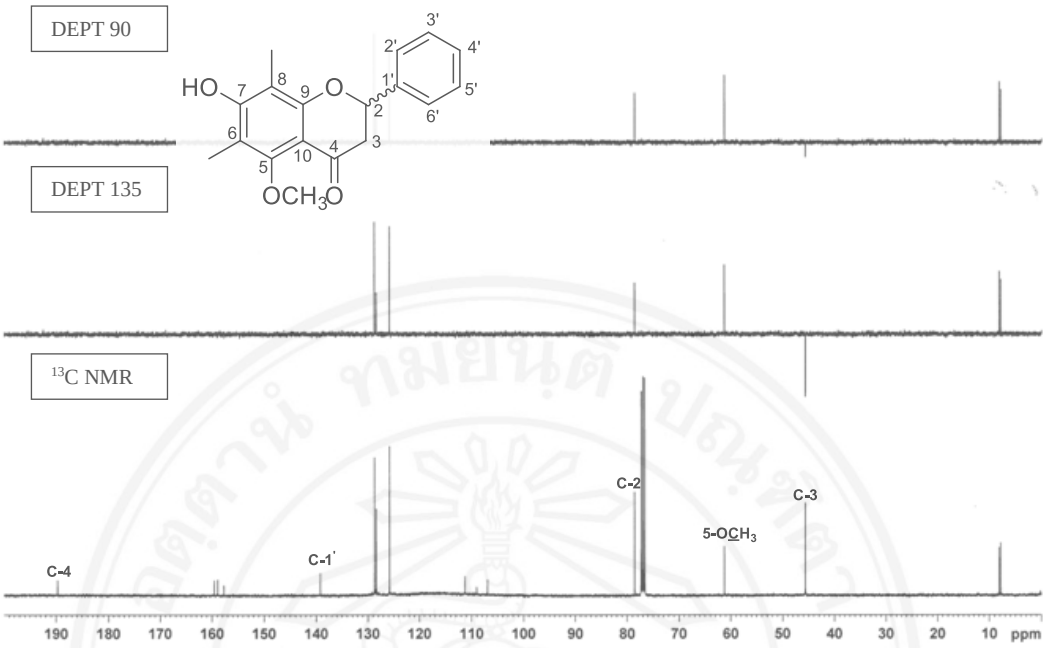


Figure 3.29 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**139**)

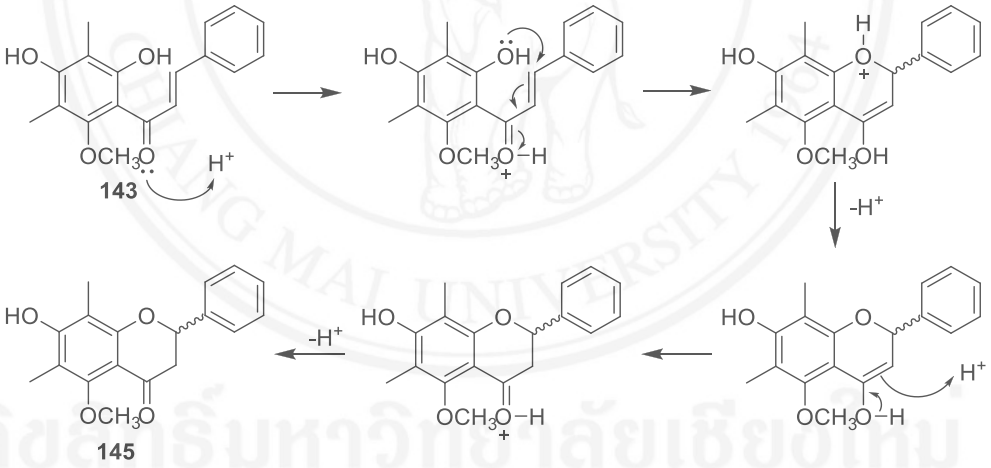
Table 3.18 ^{1}H and ^{13}C NMR data of 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**139**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [76] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [80] (ppm)
2	5.41 (*dd*, 13.0, 3.0)	5.41 (*dd*, 12.8, 3.2)	78.6	78.6
3	2.83 (*dd*, 16.7, 3.1)	2.83 (*dd*, 16.7, 3.2)	45.7	45.7
	2.97 (*dd*, 16.7, 13.0)	2.97 (*dd*, 16.7, 12.8)		
4	-	-	189.7	189.7
5	-	-	158.9	158.8
6	-	-	111.3	111.2
7	-	-	159.6	159.6
8	-	-	107.0	106.9
9	-	-	157.7	157.7
10	-	-	109.1	109.1
1′	-	-	139.2	139.2

Table 3.17 (Continued)

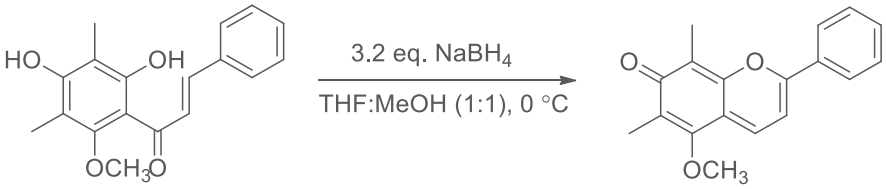
Positions	δ_H (Mult., *J* in Hz)	δ_H [76] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [80] (ppm)
2′	7.35–7.50 (*brm*)	7.35–7.50 (*brm*)	128.7	128.7
3′			128.4	128.4
4′			125.8	125.8
5′			128.4	128.4
6′			128.7	128.7
5-OCH_3	3.81 (*s*)	3.81 (*s*)	61.2	61.3
6-CH_3	2.14 (*s*)	2.14 (*s*)	7.8	7.9
7-OH	5.46 (*brs*)	5.35 (*brs*)	-	-
8-CH_3	2.14 (*s*)	2.14 (*s*)	8.1	8.1

Recorded in $CDCl_3$



Scheme 3.7 Mechanism of cyclization reactions of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (**128**)

3.3.2 5-Methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (140)



128 **140**

34% yield

The reduction of chalcone **128** with sodium borohydride in the ratio of tetrahydrofuran and methanol (1:1) as solvent at 0 °C, and purification by flash column chromatography (silica gel) using EtOAc:hexane (3:7) as eluent provided *5-methoxy-6,8-dimethyl-2-phenyl-7*H-*1-benzopyran-7-one* (**140**) in 34% yield and 55% conversion from the starting material. Compound **140** was obtained as a red solids with a melting point 78.5–79.8 °C and analysed for $C_{18}H_{17}O_3$ by means of ESIMS measurement on the $[M+H]^+$ ion (*m/z*) 281.1180 calcd for 281.1178. The IR absorption showed C=O stretching band at 1509 cm^{-1} and the band at 1131 cm^{-1} indicated the presence of ether group. Information from the ^1H NMR spectrum exhibited singlet signals of one methoxyl and two methyl protons at 3.85, 2.17 and 2.24 ppm which indicated 5-OCH_3, 6-CH_3 and 8-CH_3, respectively. Furthermore, the structural of compound **140** was confirmed by ^1H-^1H COSY spectrum (Figure 3.42), which displayed signals at δ 6.86 (1H, *d*, *J* = 7.4 Hz, H-3) correlated with proton H-4 (1H, *d*, *J* = 7.4 Hz) at 7.67 ppm as shown in Table 3.19.

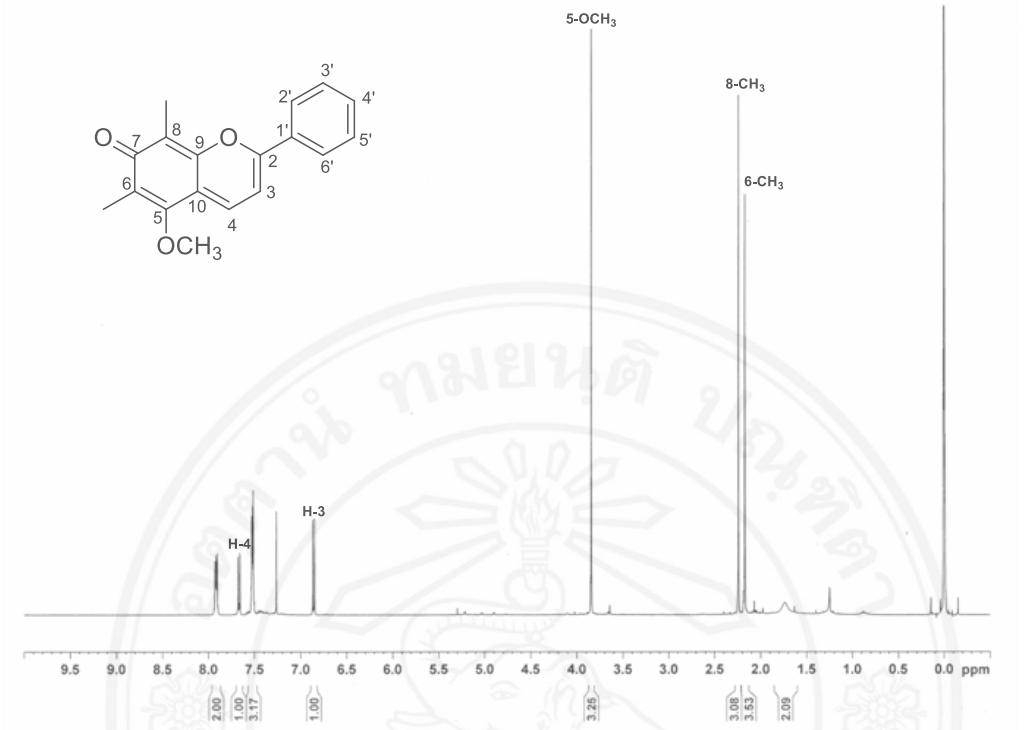


Figure 3.30 ^{1}H NMR spectrum of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

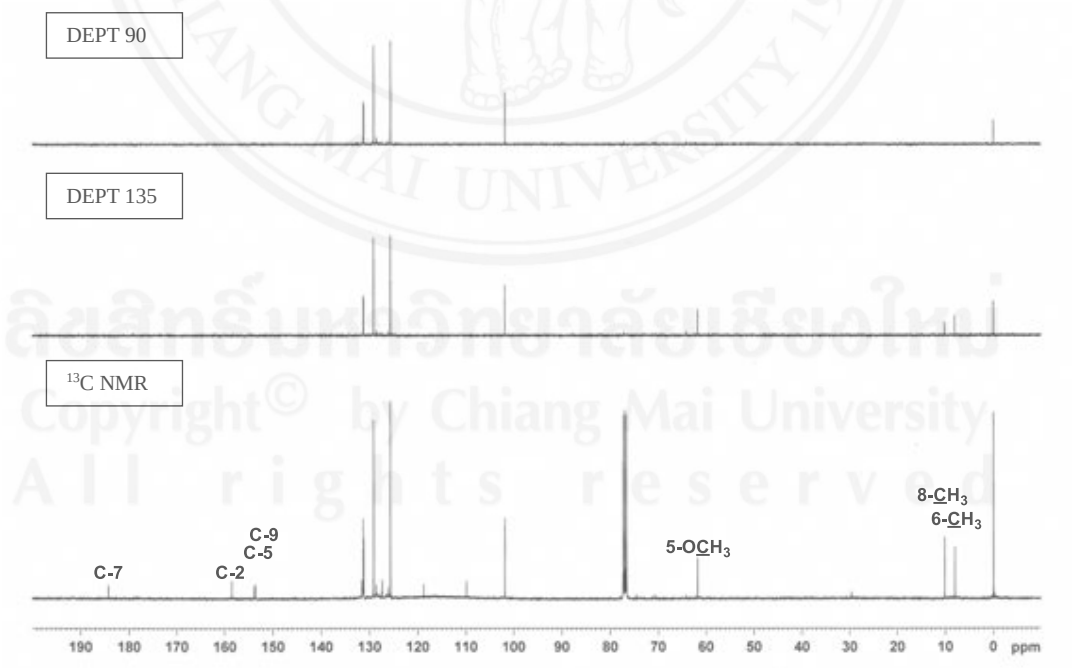


Figure 3.31 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

Moreover, this compound was approved the structure by HMQC data that indicates the correlation between each proton and carbon. For instance, H-3 proton at 6.86 ppm was coupled with C-3 at 101.9 ppm, H-4 proton at 7.67 ppm accorded with C-4 at 131.3 ppm, three protons of methoxyl group at 3.85 ppm correlated with 5-O<u>C</u>H$_3$ at 61.8 ppm, and six protons of methyl group at 2.17 and 2.24 ppm associated with 6-<u>C</u>H$_3$ and 8-<u>C</u>H$_3$, respectively.

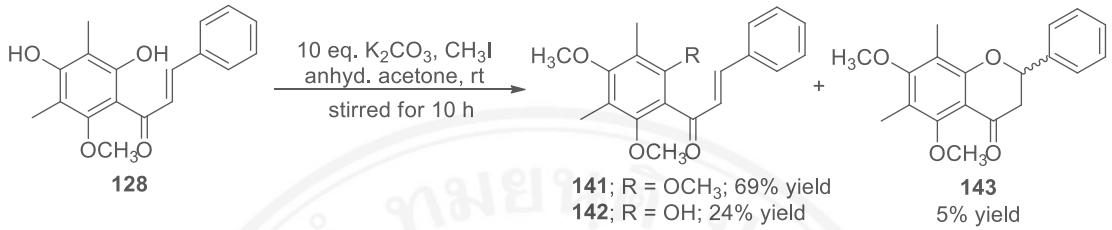
Analysis of HMBC spectrum revealed the correlations of proton signal at 7.67 (*d*, *J* = 7.4 Hz) of methine proton H-4 with the carbon signals at 158.5 (C-2), 101.9 (C-3), 153.7 (C-5), and 118.8 (C-10), the proton signal at 6.86 (*d*, *J* = 7.4 Hz, H-3) correlated with the carbon signals at 158.5 (C-2), 131.3 (C-4), 118.8 (C-10), and 131.5 (C-1′). In addition, the singlet signal at 3.85 of methoxyl proton showed correlation with the carbon signal at δ 153.7 (C-5).

Table 3.19 ^{1}H and ^{13}C NMR data of 5-methoxy-6,8-dimethyl-2-phenyl-7*H*-1-benzopyran-7-one (**140**)

Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)	Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)
2	-	158.5	1′	-	131.5
3	6.86 (*d*, 7.4)	101.9	2′	7.92 (*m*)	131.2
4	7.67 (*d*, 7.4)	131.3	3′		125.7
5	-	153.7	4′	7.52 (*m*)	129.2
6	-	109.9	5′		125.7
7	-	184.3	6′	7.92 (*m*)	131.2
8	-	127.4	5-OCH$_3$	3.85 (*s*)	61.8
9	-	153.6	6-CH$_3$	2.17 (*s*)	8.0
10	-	118.8	8-CH$_3$	2.24 (*s*)	10.1

Recorded in $CDCl_3$

3.3.3 2',4',6'-trimethoxy-3',5'-dimethylchalcone (141), 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (142) and 5,7-dimethoxy-6,8-dimethylflavanone (143)



The methylation of DMC with potassium carbonate and methyl iodide at room temperature gave three adducts, chalcones **141**, **142**, and flavanone **143** in 69, 24, and 5% yields, respectively. The mechanism for synthesis of these flavonoids as shown in Scheme 3.8.

Compound **141** was obtained as a brown oils and exhibited a molecular ion peak in ESIMS at *m/z* 349.1418 $[M+Na]^+$ (calculation 349.1416, positive ion mode), corresponding to a molecular formula of $C_{20}H_{22}O_4Na$. The IR spectrum showed absorption band of conjugated carbonyl group at 1683 cm^{-1} and aromatic nucleus at 1633 cm^{-1}.

The ^1H NMR spectrum of compound **141** in $CDCl_3$ exhibited two doublet signal of the β-proton at δ 7.04 and the α-proton at δ 7.37 ppm as a *trans*-disubstituted double bond with *J* coupling 16.1 Hz. In addition, the protons of three methoxy groups presented signal at δ 3.68, 3.68 and 3.73 (9H, *s*, 2′,6′,4′-OC$\underline{H}_3$ respectively), two methyl groups at δ 2.21 (6H, *s*, 3′- and 5′-C$\underline{H}_3$) as shown in Figure 3.32. Furthermore by the analysis of the ^{13}C NMR spectrum in combination with the DEPT spectra, compound **141** was proved to contain one carbonyl carbon, two methyl carbons, three methoxyl carbons, seven methine carbons and seven quarternary carbons. The data are as shown in Figure 3.33 and Table 3.20.

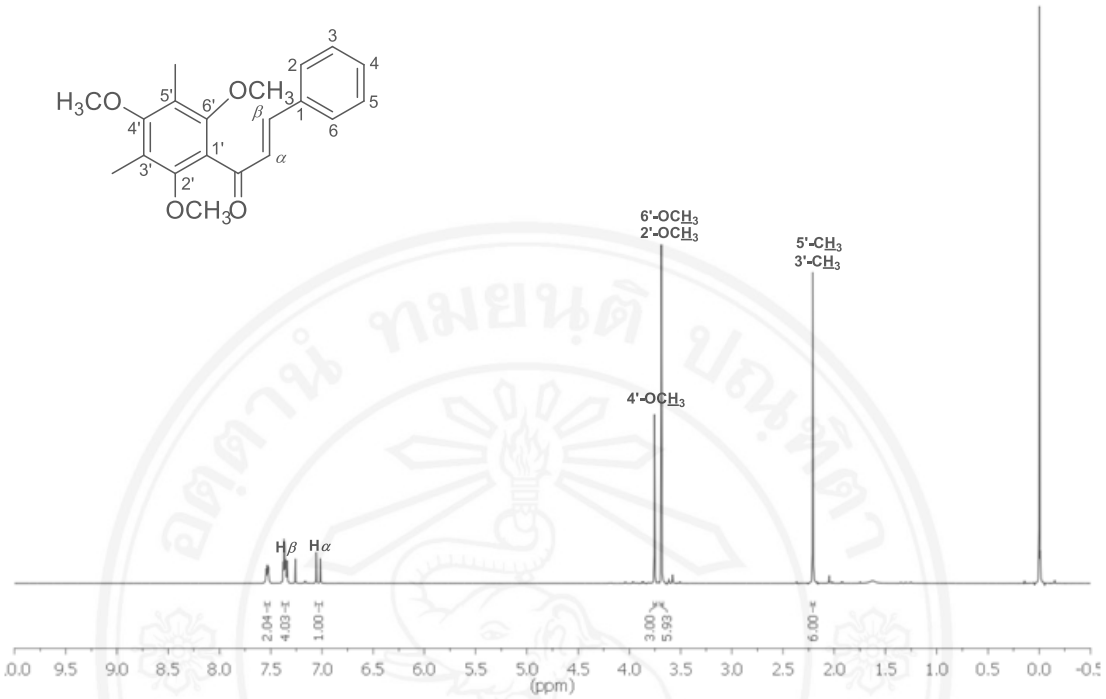


Figure 3.32 ^{1}H NMR spectrum of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

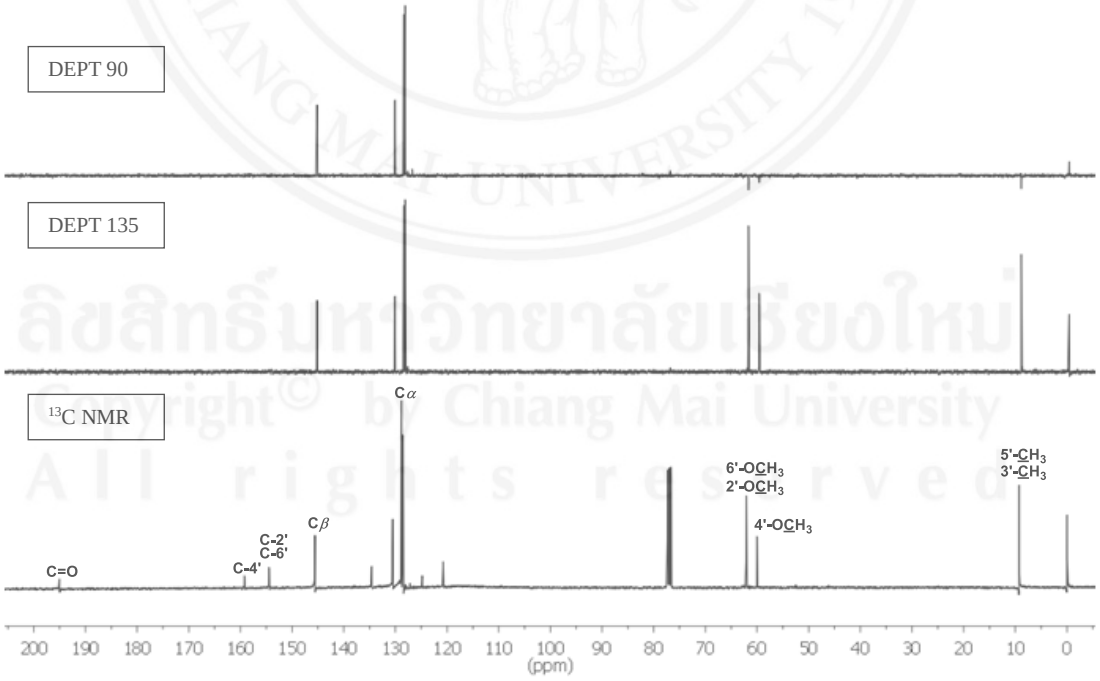


Figure 3.33 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

Table 3.20 ^{1}H and ^{13}C NMR data of 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**)

Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)	Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)
1	-	134.6	2′	-	154.5
2	7.54 (*m*)	128.6	3′	-	120.8
3	7.36 (*m*)	128.8	4′	-	159.2
4		130.5	5′	-	120.8
5		128.8	6′	-	154.5
6	7.54 (*m*)	128.6	2′-OCH_3	3.68	62.1
β	7.37 (*d*, 16.1)	145.6	3′-CH_3	2.21	9.2
α	7.04 (*d*, 16.1)	128.4	4′- OCH_3	3.73	60.0
C=O	-	195.1	5′-CH_3	2.21	9.2
1′	-	124.8	6′-OCH_3	3.68	62.1

Recorded in $CDCl_3$

Compound **142** was obtained as a yellow oils and exhibited a molecular ion peak in ESIMS at *m/z* 335.1259 $[M+Na]^+$ (calculation 335.1259, positive ion mode), corresponding to a molecular formula of $C_{19}H_{20}O_4Na$. The IR spectrum of **142** showed a characteristic C=O stretching band of the conjugated carbonyl system at 1648 cm^{-1}. The absorption band at 1586 cm^{-1} was assigned to the C=C stretching of nucleus and absorption band at 3481 cm^{-1} was referred to the hydroxyl group.

The 1D NMR spectra (^{1}H and ^{13}C) of compound **142** in $CDCl_3$ displayed the signals of dimethoxyl groups as two singlet signal at δ 3.76 and 3.66 (4′- and 6′-O<u>C</u>H_3). Dimethyl groups appeared signals at δ 2.17 and 2.18 (3′- and 5′-C<u>H</u>$_3$), and hydroxyl proton (2′-O<u>H</u>) for hydrogen bonding of carbonyl group (C=O) indicated signal at δ 13.09 ppm. Furthermore the β- and α-proton showed doublet signals of an α,β-unsaturated ketone at δ 7.98 and 7.86 ppm with *J* coupling = 15.7 Hz (Figure 3.34). Moreover, this compound was confirmed the structural by DEPT-135 and DEPT-90 spectra and compare with the literature values as shown in Table 3.21 and Figure 3.35. Compound **142** has been reported from the roots of *P. carniconnectivum* C.DC. (Piperaceae) as 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone [75] (Table 3.21).

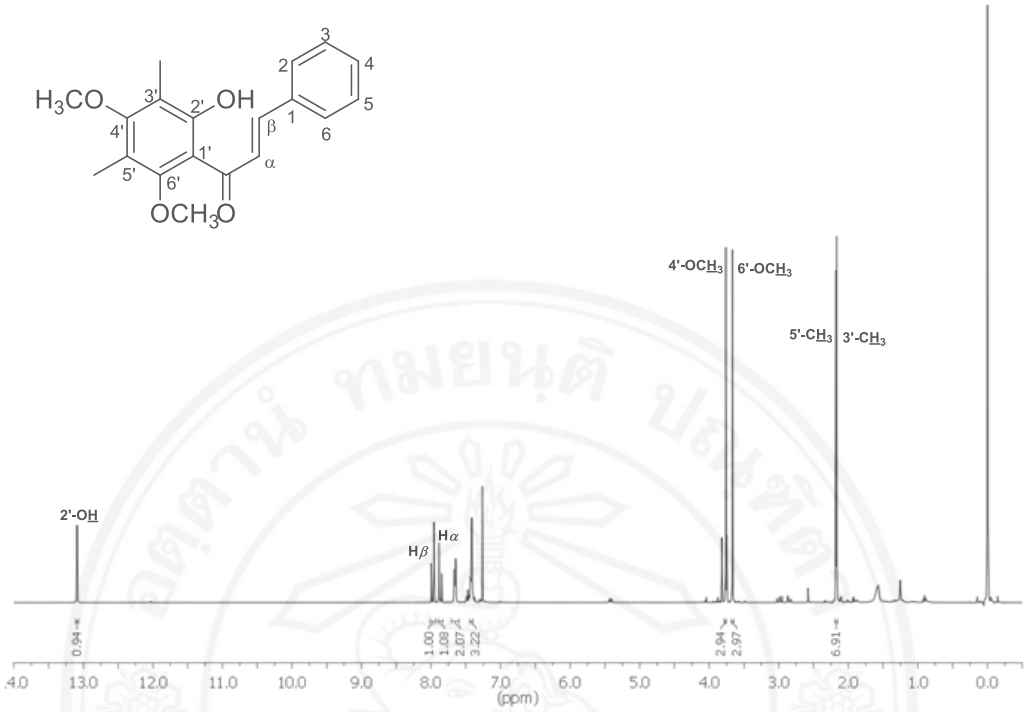


Figure 3.34 ^{1}H NMR spectrum of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

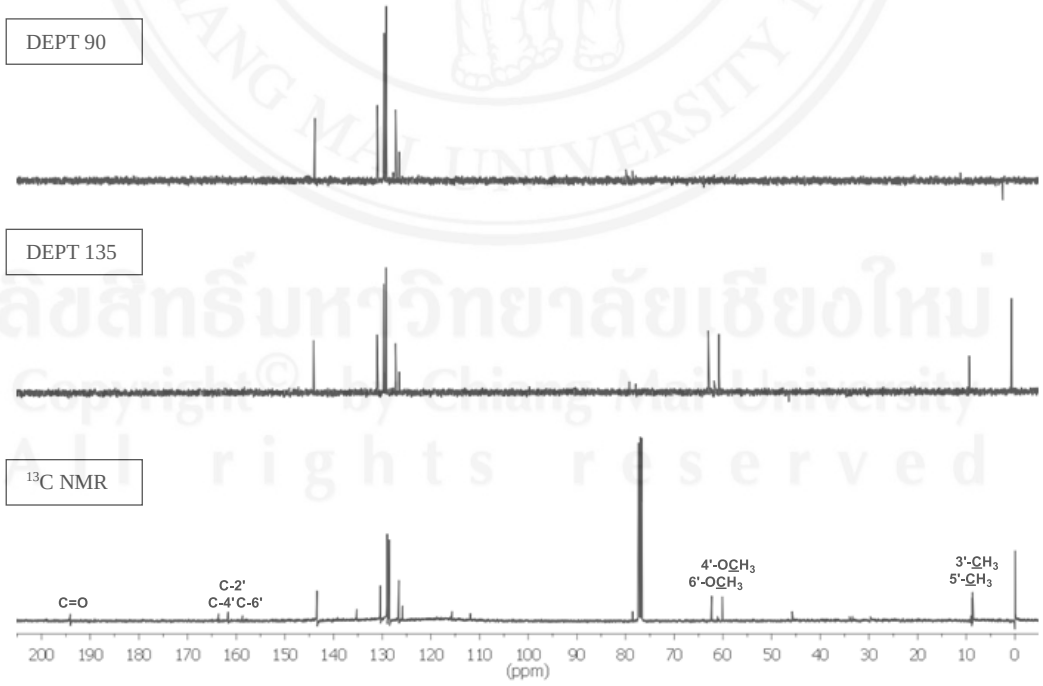


Figure 3.35 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

Table 3.21 ^{1}H and ^{13}C NMR data of 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethylchalcone (**142**)

Positions	δ_H (Mult., *J* in Hz)	δ_H [75] (Mult., *J* in Hz)	δ_C (ppm)	δ_C [75] (ppm)
1	-	-	135.2	135.2
2	7.65 (*m*)	7.65 (*m*)	128.5	128.5
3	7.42 (*m*)	7.48 (*m*)	129.0	128.9
4	7.42 (*m*)	7.46 (*m*)	130.4	130.4
5	7.42 (*m*)	7.48 (*m*)	129.0	128.9
6	7.65 (*m*)	7.65 (*m*)	128.5	128.5
β	7.98 (*d*, 15.7)	7.97 (*d*, 15.7)	143.4	143.4
α	7.86 (*d*, 15.7)	7.80 (*d*, 15.7)	125.8	125.9
C=O	-	-	194.1	194.1
1′	-	-	111.9	111.9
2′	-	-	161.6	161.6
3′	-	-	115.7	115.7
4′	-	-	163.6	163.6
5′	-	-	115.7	115.7
6′	-	-	158.7	158.7
2′-OH	13.09 (*s*)	13.09 (*s*)	-	-
3′-CH_3	2.17 (*s*)	2.17 (*s*)	8.7	8.7
4′- OCH_3	3.76 (*s*)	3.76 (*s*)	60.1	60.1
5′-CH_3	2.18 (*s*)	2.18 (*s*)	8.8	8.8
6′-OCH_3	3.66 (*s*)	3.66 (*s*)	62.3	62.3

Recorded in $CDCl_3$

Compound **143** was obtained as a white solids with a melting point 97.8–98.4 °C and exhibited a molecular ion peak in ESIMS at *m/z* 335.1262 $[M+Na]^+$ (calculation 335.125995, positive ion mode), corresponding to a molecular formula of $C_{19}H_{20}O_4Na$. The IR spectrum showed C=O stretching band at 1649 cm^{-1} and the band at 1108 cm^{-1} suggested the presence of ether unit.

The 400 MHz ^{1}H NMR ($CDCl_3$) spectrum of compound **143** indicated that compound **143** was a flavanone skeleton. Its showed doublet of doublets signals at 5.42 (J = 13.1, 3.0 Hz) for H-2 proton, and two protons for H-3 splitting pattern observed as two signals which produce doublet of doublets at δ 2.98 (J = 16.6, 13.1 Hz) and 2.86 (J = 16.6, 3.1 Hz) ppm could be assigned as flavanone **143**. In addition, two singlet signals at δ 3.75 and 3.82 showed dimethoxyl protons (5,7-OC$\underline{H}_3$) and one singlet signal at δ 2.16 ppm observed dimethyl protons (6,8-C$\underline{H}_3$) as shown in Table 3.22 and Figure 3.36. Furthermore the DEPT-135, DEPT-90 and ^{13}C NMR spectra showed especial signals, for example, C-4 carbon for carbonyl group indicated peak at δ 192.0, C-3 methylene carbon displayed peak at δ 45.8, and three oxygenated aromatic carbons showed peaks at δ 163.4, 159.7, and 157.7 ppm as deduced C-7, C-5, and C-9, respectively (Figure 3.37 and Table 3.22).

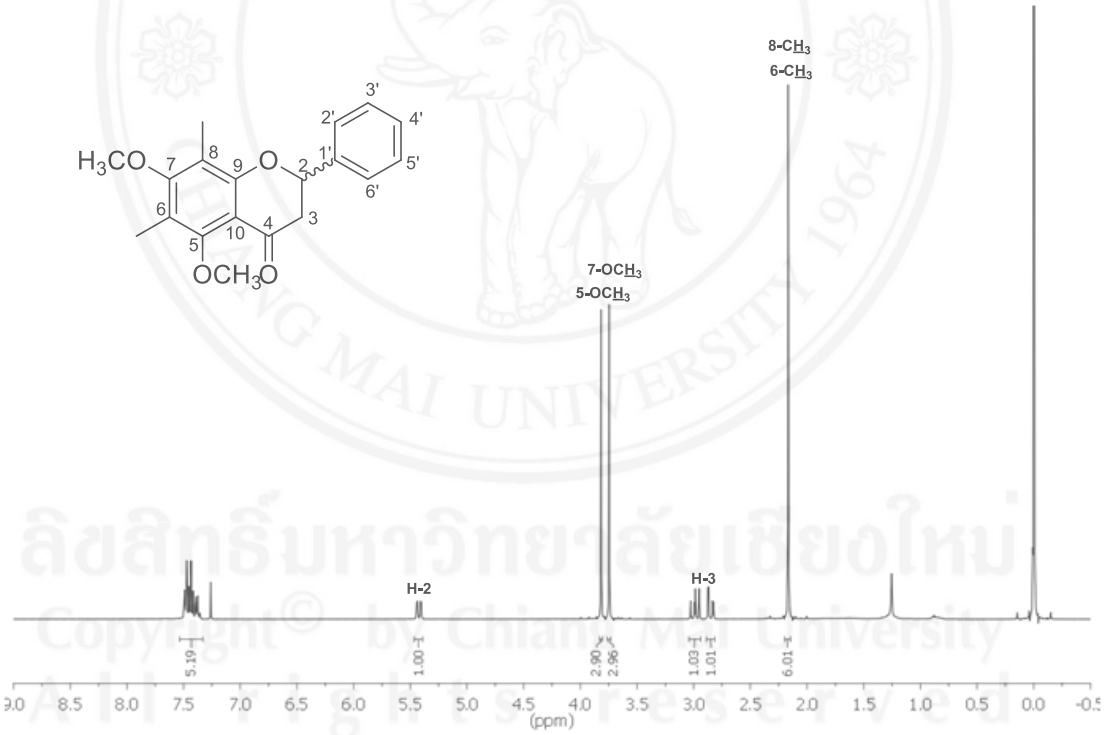


Figure 3.36 ^{1}H NMR spectrum of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

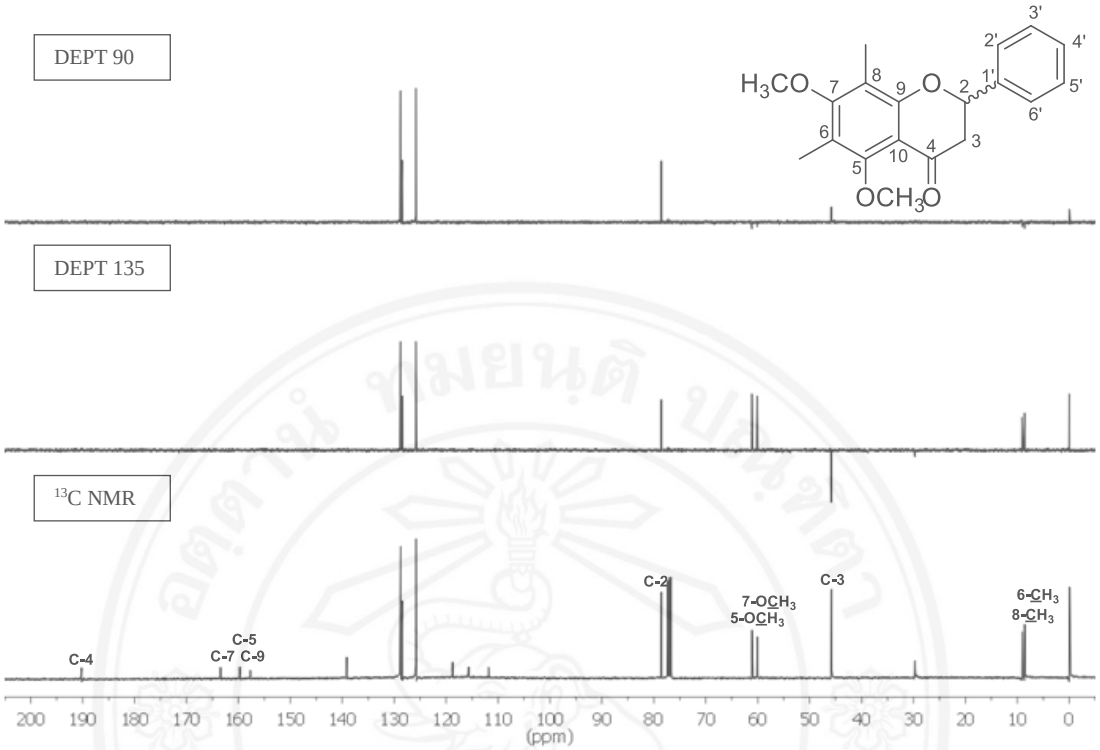
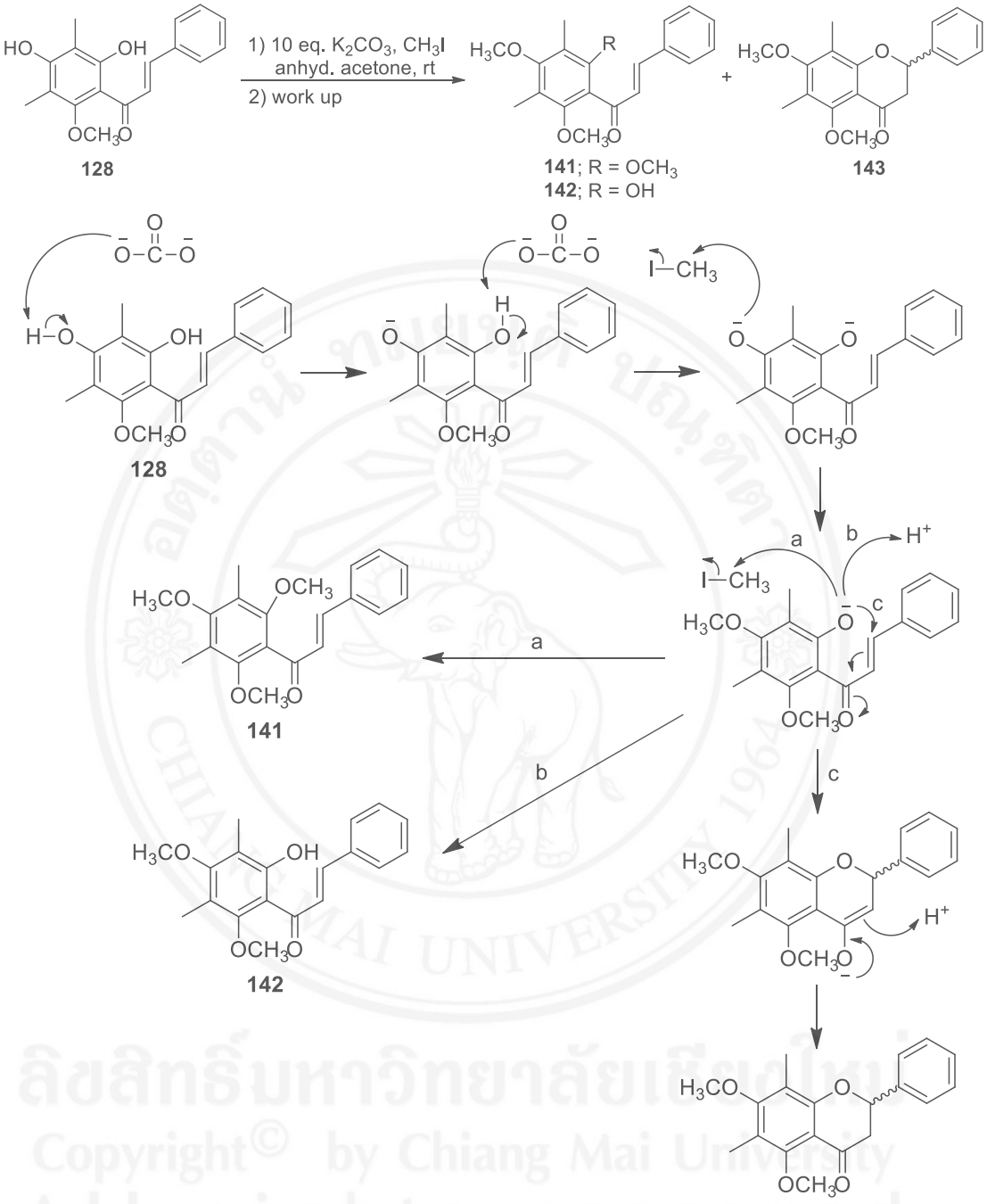


Figure 3.37 DEPT 90, DEPT 135 and ^{13}C NMR spectra of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

Table 3.22 ^{1}H and ^{13}C NMR data of 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)	Positions	δ_H (Mult., *J* in Hz)	δ_C (ppm)
2	5.42 (*dd*, 13.1, 3.0)	78.5	1′	-	139.1
3	2.85 (*dd*, 16.6, 3.1)	45.8	2′	7.35–7.50 (*brm*)	128.7
	2.99 (*dd*, 16.6, 13.1)		3′		125.8
4	-	190.2	4′		128.4
5	-	159.7	5′		125.8
6	-	118.7	6′		128.7
7	-	163.4	5-OCH$_3$	3.82 (*s*)	61.1
8	-	111.8	6-CH$_3$	2.17 (*s*)	8.6
9	-	157.7	7-OCH$_3$	3.75 (*s*)	60.1
10	-	115.6	8-CH$_3$	2.17 (*s*)	9.1

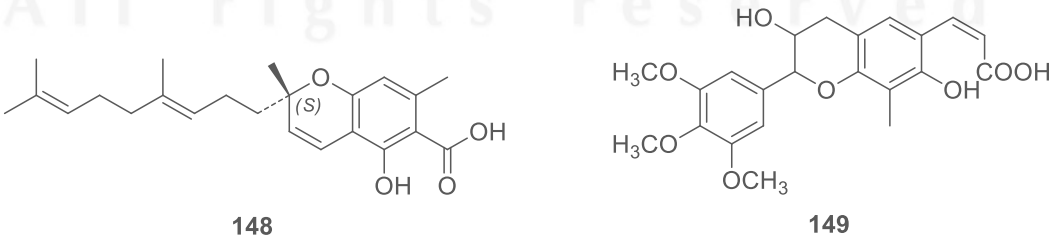
Recorded in $CDCl_3$



Scheme 3.8 Mechanism of synthesis of flavonoids; 2',4',6'-trimethoxy-3',5'-dimethylchalcone (**141**), 2'-hydroxy-4',6'-dimethoxy-3',5'-dimethyl-chalcone (**142**) and 5,7-dimethoxy-6,8-dimethylflavanone (**143**)

All pure compounds isolated from *C. nervosum* var. *paniala* seeds and synthetic DMC derivatives (**139**-**142**) obtained in the present investigation were evaluated against cancer cells and anti-HIV-1 RT activity (Table 3.23). Among these compounds, DMC (**128**) and **140** showed cytotoxicity to P-388, KB, MCF-7, A549, ASK and Hek293 cell lines, compounds **141** and **142** exhibited active on cancer cell, on the other hand, hariganetin (**138**) and **139** showed inactive on cell lines. However, compound **142** exhibited high cytotoxicity to all tested cancer cell lines when compared with ellipticine. The result of the present study also revealed the anti-HIV-1 RT activity tested compounds. It was found that, compound **140** elicited a significant high inhibitory effect on HIV-1 RT with the values of 93.40 % inhibition.

From the result that shown in Table 3.23, it was found that DMC and theirs derivatives provide different potencies in *vivo* depending on structural efficiency. Compound **142** has high potential when compared with compound **141** because of the importance of substitution on aromatic ring between the substituent at position 2′ and 4′-OH on aromatic moiety. The position 2′-OH was necessary for potent against cancer activity more than position 4′-OH, in case of unsubstituted analogues **141**, which replaced by OCH_3, showed less active in these biological activity. The benzopyran-containing compounds are found to have significant anti-HIV compared to references. Daurichromenic acid (**148**) has shown highly potent anti-HIV activity in acutely inflected H9 cells with an EC_{50} of 0.00567 μg/ml and a therapeutic index (TI) of 3710 [81]. The anti-HIV activity *in vitro* of melloapeltic acid (**149**) was assayed on MT-4 cell with an IC_{50} value of 3.2 μg/ml and TI value of 3.5. In general, TI > 5.0 is considered to denote significant activity [82]. Therefore, compound **140** consist of a group of benzopyran compounds are effective against HIV than other compounds.



148 149

Figure 3.38 Structures of daurichromenic acid (**148**) and melloapeltic acid (**149**)

Table 3.23 Cytotoxicity of flavonoids against cancer cell lines and anti-HIV-1activity of isolated compounds from *C. nervosum* var. *paniala* seeds and derivatives DMC

Compounds	Cytotoxicity (ED_{50}, μg/mL)							HIV-1 RT assay % Inhibition
	P-388	KB	HT29	MCF-7	A549	ASK	HeK293	at 200 μg/mL
DMC (**128**)	10.31	15.90	NR	14.51	13.04	9.00	2.39	67.46 (M)
Hariganetin (**138**)	NR	NR	NR	NR	NR	NR	NR	64.05 (M)
Compound **139**	NR	NR	NR	NR	NR	NR	NR	43.57 (W)
Compound **140**	10.15	14.36	NR	3.42	15.74	18.72	11.64	93.40 (VA)
Compound **141**	2.01	9.09	9.25	2.68	7.85	7.69	2.63	52.11 (M)
Compound **142**	2.06	3.12	3.56	2.51	3.28	4.79	2.15	-17.86 (IA)
Ellipticine (Positive control)	0.56	0.53	0.61	0.51	0.48	0.38	0.49	NT

Cytotoxic assay: ED_{50} less than 20 μg mL^{-1} were considered active for extracts and less than 4 μg mL^{-1} for pure compounds. P-388: murine lymphocytic leukemia, KB: human oral nasopharyngal carcinoma, HT29: human colon cancer, MCF-7: human breast cancer, A549: human lung cancer, ASK: rat glioma cell, HeK298: noncancerous human embryonic kidney cell, HIV-1 RT: human immunodeficiency virus-1 reverse transcriptase, NR: no response (ED_{50}>20 μg mL^{-1}), NT: not test, VA: very active, M: medium active, W: weakly active, IA: inactive.