CHAPTER III

RESULTS AND DISCUSSION

3.1 Structural Elucidation of the Isolated Compounds

3.1.1. Compound (1)

Compound (1) was obtained from the hexane parts which was separated on the silica gel column. The oil was removed from solid by washing with *n*-hexane and then the solid residue was recrystallized from a mixture of chloroform and hexane to give white needle crystals with the Rf value 0.45 (1:1 hexane : chloroform as developing solvent and using silica gel TLC plate)

This compound was soluble in chloroform and slightly soluble in *n*-hexane. The color tests of this compound indicated that this compound had a steroidal skeleton and unsaturation part in molecule.

The IR spectrum (Fig. 1) gave the absorption bands of secondary alcohol at 3430 and 1050 cm^{-1} and bands at 1660-1640 cm^{-1} due to the C=C stretching vibration. The absorption bands at 840 and 800 cm^{-1} (C-H out of plane bending) coincided with the characteristic absorption peaks of trisubstituted olefin. The IR absorption band assignments of this compound were presented in Table 3.1 [47,48].

Table 3.1 The IR absorption band assignments of Compound (1)

Frequency (cm^{-1})	Band type	Tentative assignments
3430	b	O-H stretching vibration
2960-2840	s	C-H stretching vibration of $-CH_3$, $-CH_2-$
1650	w	C=C stretching vibration
1460	m	C-H bending vibration of $-CH_3$, $-CH_2-$
1380	m	C-H in plane bending vibration of trisubstituted of olefin
1050	m	C-O stretching vibration of secondary alcohol
970,950	m	C-H out of plane bending vibration of trans configuration
840,800	w	C-H out of plane bending vibration of trisubstituted olefin

The results of the color tests and the IR spectral data supported a steroidal structure of this compound.

The ^1H NMR spectrum ($CDCl_3$) of Compound (1) (Fig. 2) showed the signals at 0.70-1.06 ppm., which were the signals of angular methyl groups of C-18 and C-19 and methyl groups at side chain which were C-21, C-26, C-27 and C-29. The signals at 1.51-2.30 ppm. exhibited methylene group ($-CH_2-$) and methylnic group (CH) of

steroids. The proton of hydroxyl group showed the multiplet signal at 3.46 ppm. while the double of doublet at 5.08 ppm. was the signal of disubstituted vinyl protons (H-22 and H-23). The last signal at 5.32 ppm. was the signal of trisubstituted vinyl proton (H-6).

The ^{13}C NMR spectrum ($CDCl_3$) (Fig. 3) showed the signals which corresponded to the ^{13}C NMR spectrum of β-sitosterol and stigmasterol as showed in Table 3.2 [49].

Table 3.2 The ^{13}C NMR chemical shifts of β-sitosterol, stigmasterol and Compound (1)

Carbon position	Chemical shift (ppm.)		
	β-sitosterol	stigmasterol	Compound (1)
1	37.1	37.4	37.29
2	31.8	31.7	31.60
3	71.9	71.8	71.69
4	42.4	42.4	42.28
5	140.9	140.0	140.76
6	121.8	121.7	121.64
7	32.0	31.9	31.87
8	32.0	31.9	31.87
9	50.3	50.3	50.18

(continued)

Table 3.2 (continued)

Carbon position	Chemical shift (ppm.)		
	β-sitosterol	stigmasterol	Compound (1)
10	36.6	36.6	36.48
11	21.1	21.1	21.09
12	39.9	39.8	39.73
13	42.4	42.4	42.28
14	56.8	57.0	56.79
15	24.3	24.4	24.29
16	28.2	28.9	28.24 , 28.89
17	56.2	56.0	55.98
18	11.9	12.2	11.83 , 12.21
19	19.4	19.4	19.36
20	36.2	40.5	36.15 , 40.43
21	19.1	21.1	18.99 , 21.09
22	34.0	138.4	33.99 ,138.27
23	29.3	129.4	29.22 ,129.28
24	50.3	51.3	50.21
25	26.2	31.9	26.13 , 31.87
26	18.8	19.0	18.99
27	19.8	21.1	19.79 , 21.20
28	23.1	25.4	23.09 , 25.37
29	11.9	12.0	11.83 , 12.04

The MS spectrum (Fig. 4) showed the expected molecular ion peak of β-sitosterol at m/e 414.0 and also revealed the fragmentation pattern of C-29 steroids. However, two significant peaks at m/e 412.0 and m/e 400.0 could not be accounted for the β-sitosterol structure. The mass spectrum pattern of this compound indicated that this compound was a mixture compound of steroids generally found in most plants. However, this mixture could not be separated eventhough the adsorbent of the column and the developing solvent system were varied. The possible mass fragmentation pattern of Compound (1) was presented in Scheme 3.1 [50].

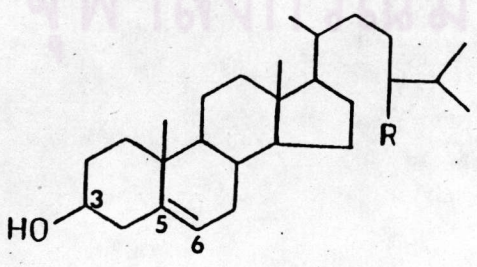
The presence of β-sitosterol in plants is frequently accompained by one or more steroids [51] such as stigmasterol and campesterol. When compared the GLC analysis data of Compound (1) (Fig. 5) with those of the authentic campesterol, stigmasterol, chloresterol and β-sitosterol, it indicated that Compound (1) corresponded to β-sitosterol, stigmasterol and campesterol as showed in Table 3.3.

Table 3.3 The comparison of GLC retention times between Compound (1) and authentic steroids

Compound	Retention time (min)
chloresterol	14.43
campesterol	18.23
stigmasterol	19.63
β-sitosterol	22.09
Compound (1)	18.37
	19.32
	21.98

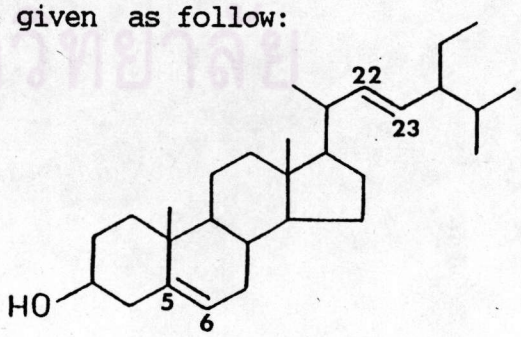
OV-1 2% column, col. temp. 260 °C , injection temp. 290 °C detector temp. 290 °C, N_2 50 ml/min, injection volume 1 μl

The results above indicated that Compound (1) was a mixture of β-sitosterol, stigmasterol and campesterol. The structure of these three steroids are given as follow:



R=Et , β-sitosterol

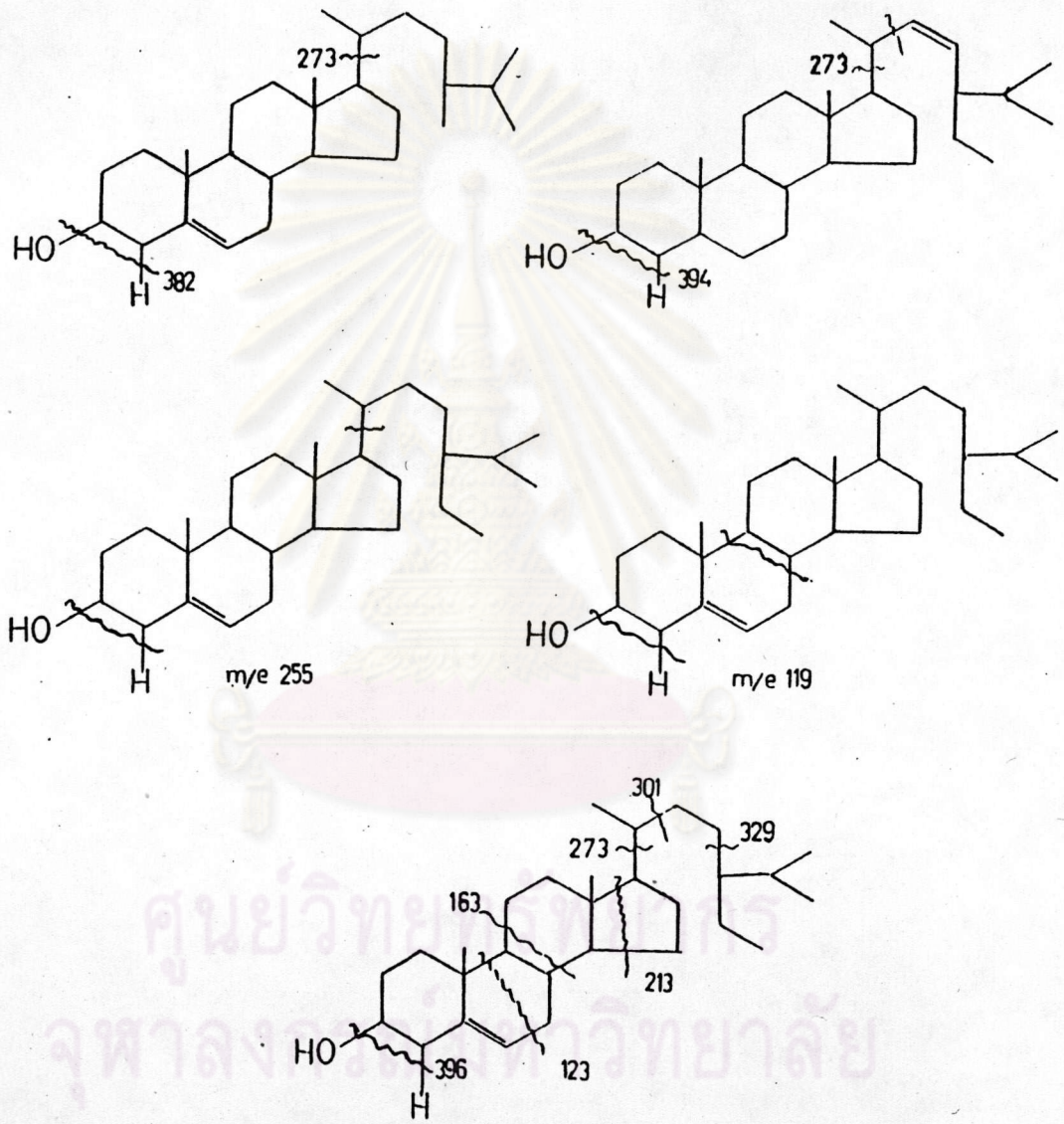
R=Me , campesterol



stigmasterol

Compound (1)

Scheme 3.1 The possible mass fragmentation pattern of Compound (1)



3.1.2 Compound (2)

Compound (2) was collected from the hexane parts , using silica gel column. After purification by recrystallization from hexane, pale yellow needle crystals were obtained. This compound showed a single spot with the Rf value 0.68 (solvent 1:19 *n*-hexane-chloroform using silica gel TLC plate) and m.p. 103.0-104.0 °C.

This compound gave negative result with Liebermann-Burchard's reagent. It gave a positive results with 2,4 DNP and decolorized Br_2 in CCl_4 reagent. These results indicated that it was not composed of steroidal or triterpenoidal skeletons.

The IR spectrum (Fig. 6) gave the absorption band of aromaticity at 3070 cm^{-1}. The vibration of carbonyl group that conjugated to double bond appeared at 1660 cm^{-1}. The peaks at 1600 and 1500 cm^{-1} were belonged to carbon-carbon double bond and C-O stretching vibration showed the absorption at 1270 and 1080 cm^{-1} The IR absorption band assignments of this compound were presented in Table 3.4 .

The ^1H NMR ($CDCl_3$) spectrum (Fig. 7) showed the signal of hydroxyl group at 12.76 ppm. The signals of olefinic protons appeared at 6.36, 5.99 and 5.14 ppm. The methoxy protons showed a signal at 3.88 ppm. and methyl protons around 1.78-1.67 ppm.

The ^1H NMR ($CDCl_3$) spectrum (Fig. 7) showed the signals which corresponded to the ^1H NMR spectrum of heteropeucenin-7-methyl ether [11] and are presented in Table 3.5.

Table 3.4 The IR absorption band assignments of Compound (2)

Frequency (cm^{-1})	Band type	Tentative assignment
3070	w	=C-H stretching vibration.
2980,2850	m	C-H stretching vibration.
1660	s	C=O stretching vibration of α,β-unsaturated ketone
1620-1500	s	C=C stretching vibration.
1270-1210	s	C-O-C asym. stretching
1080	s	C-O-C sym. stretching
1110-980	s	C-H in plane bending of aromatic
850-720	m	C-H out of plane bending

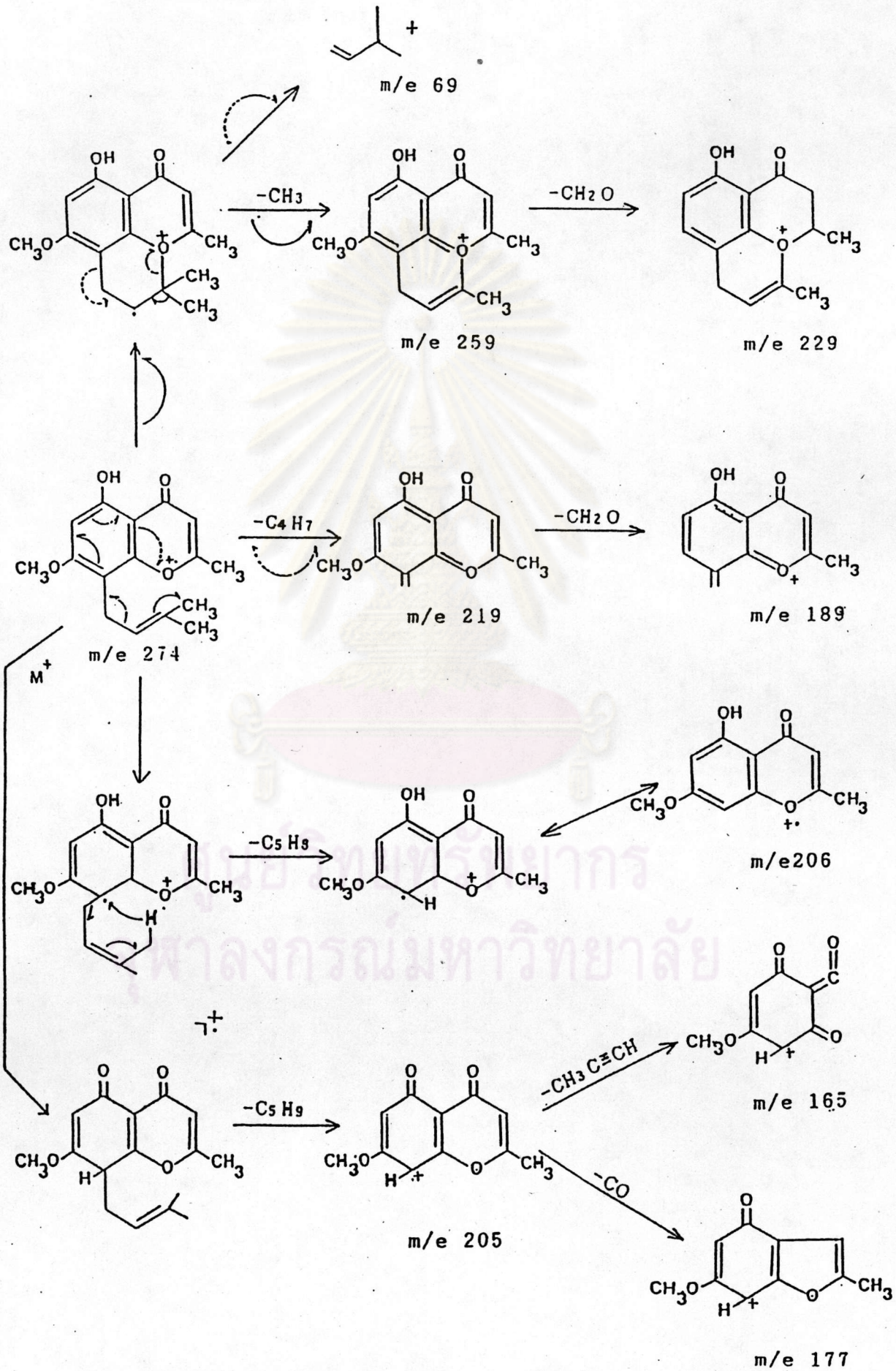
The ^{13}C NMR ($CDCl_3$) spectrum (Fig.8) gave the important carbon signals which could be assigned as follow : the carbonyl carbon was located at 182.89 ppm., the olefinic and aromatic carbon showed the signals around 166.69-104.66 ppm. Methoxy carbon was assigned at 55.96 ppm. and other peaks around 25.73 ppm. to 17.76 ppm. were assigned to geminal dimethyl carbon.

Table 3.5 The ^1H NMR chemical shifts of heteropeucenin-7-methyl ether and Compound (2)

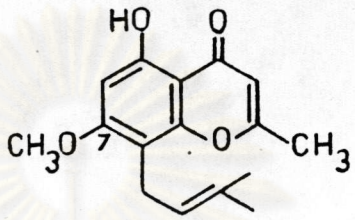
chemical shift (ppm.)	
Compound (2)	Heteropeucenin-7-methyl ether
1.67	1.60
1.78	1.70
3.33	3.30
3.88	3.80
5.14	5.08
6.36	6.24
12.77	12.68

The MS spectrum (Fig. 10) of this compound showed the molecular ion peak at m/e 274. The molecular formula was proposed as $C_{16}H_{18}O_4$. This formula was supported by the elemental analysis data. The fragmentation ion peak at m/e 259 (M^+-15) was identified as methyl group. The m/e 219 (M^+-55) suggested the elimination of $-CH=C-(CH_3)_2$ and m/e at 177 took place when a carbonyl group was eliminated after the loss of $-CH_2-CH=C-(CH_3)_2$ (M^+-69-28). The mass fragmentation pattern of Compound (2) was presented in Scheme 3.2.

Scheme 3.2 The possible mass fragmentation pattern of Compound (2)



According to spectroscopic evidences , chemical reactions and physical properties, Compound (2) was identical to heteropeucenin-7-methylether [13]. The structure of this compound was shown below :



Compound (2)

3.1.3 Compound (3)

Compound (3) was isolated from the chloroform parts using column chromatographic technique. After purification by recrystallization from the mixture of ethanol and chloroform, the pale yellow amorphous compound, m.p. 230.0 °C (dec.), Rf value 0.60 using ethanol as a developing solvent (silica gel TLC plate was used) was obtained. This compound decolorized Br_2 in CCl_4 reagent and liberated CO_2 with 5% $NaHCO_3$ solution. This results hinted the presence of unsaturated part and carboxyl part in this molecule, respectively.

The IR spectrum (fig. 11) which was assigned in Table 3.6 revealed the characteristic aromatic stretching vibration at 3040 cm^{-1}. The carboxyl and conjugated carbonyl showed the absorption peak at 1660 and 1655 cm^{-1}, respectively. The additional band of carbon-carbon double bond appeared at 1620 cm^{-1} together with geminal dimethyl group at 1385 and 1370 cm^{-1}.

Table 3.6 The IR absorption band assignments of Compound (3)

Frequency (cm^{-1})	Band type	Tentative assignments
3040	m	C-H stretching vibration of aromatic and alkene
2980,2960	w	C-H stretching vibration of $-CH_2-$, $-CH_3$
1660	s	C=O stretching vibration of -COOH
1655	s	C=O stretching of α,β-unsaturated ketone
1620,1605	s	C=C stretching vibration of double bond
1470	m	C-H sym. stretching of $-CH_2-$ and asym. stretching of $-CH_3$
1385,1370	m	C-H bending of gem-dimethyl group
1200,1150	m	C-O-C bending of ether
820,810	m	C-H bending of aromatic

The ^1H NMR (DMSO+$CDCl_3$) spectrum (Fig. 12) exhibited the singlet signal of alkene and aromatic carbon adjacent to quarternary carbon at 6.37 ppm. and 6.62 ppm., respectively. The doublet signals at 5.67 and 6.86 ppm. (J = 9.9 Hz.) could be assigned to olefinic protons (-CH=CH-), the peak at 3.83 ppm., assigned to methoxy protons (3H) and the singlet signal at 1.44 ppm. (6H) corresponded to geminal-dimethyl group (6H).

The ^{13}C NMR (DMSO) spectrum (Fig.13) exhibited the carbonyl carbon and carboxyl carbon at 175.58 and 161.46 ppm., respectively. The singlet signals at 128.29 ppm. and 115.13 ppm. were assigned to carbon-carbon double bond (-CH=CH-). The signals of benzene ring with one substituted group on each carbon atom were at 160.14 (s), 157.85(s), 150.33(s), 102.00(s) ppm. The signal at 97.03 ppm.(s) was assigned to aromatic carbon which was not connected to any substituent group. The signals at 78.29 and 56.03 ppm. could be assigned to the carbon atom with two substituents group and methoxy carbon, respectively. The signal at 27.80 ppm. was attributed to geminal dimethyl group.

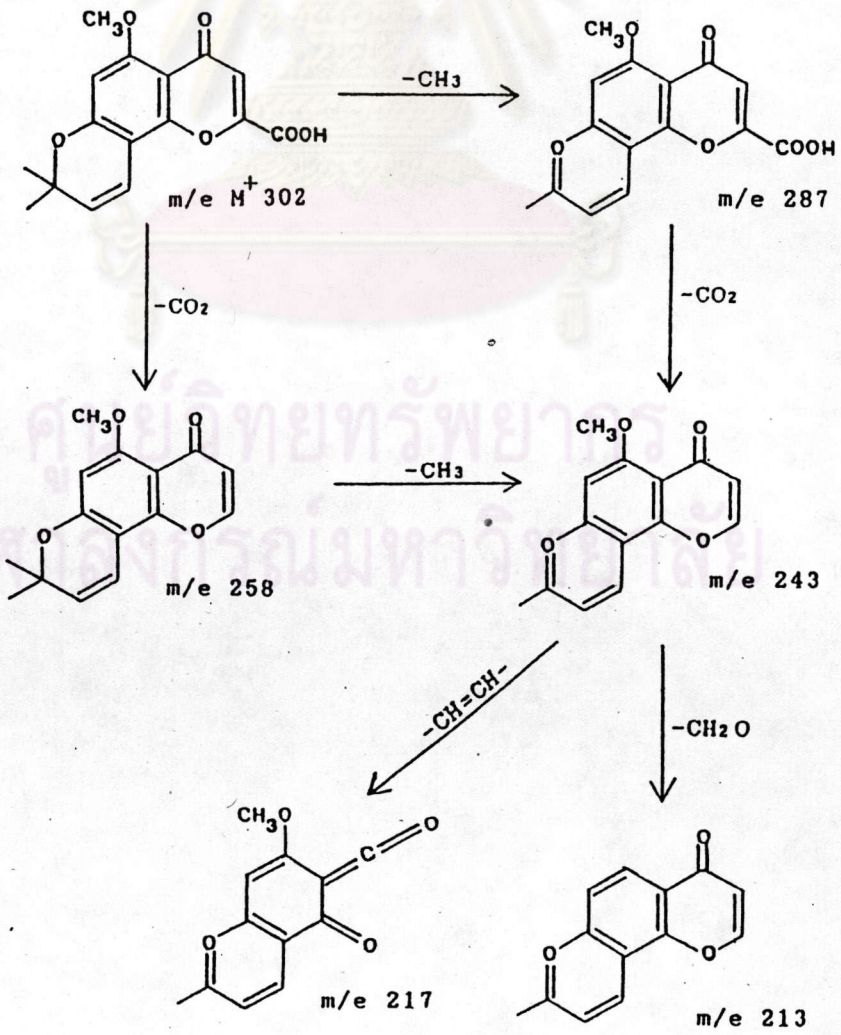
It could be concluded that Compound (3) exhibited the ^{13}C NMR chemical shifts similar to perforatic acid [12]. The ^{13}C NMR chemical shifts of Compound (3) and perforatic acid could be compared as in Table 3.7.

Table 3.7 The ^{13}C NMR chemical shifts of Compound (3) and perforatic acid.

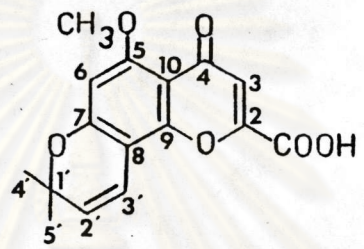
Carbon	Chemical shift (ppm.)	
	Compound (3)	Perforatic acid
2	160.14	163.2
3	114.22	117.3
4	175.85	178.6
5	150.33	153.4
6	97.03	100.1
7	157.85	160.9
8	102.00	105.1
9	152.95	156.0
10	108.81	111.9
1'	78.29	81.3
2'	115.13	118.2
3'	128.19	131.1
4'	27.80	30.9
5'	27.80	30.9
C_5-OCH_3	56.30	59.3
C_2-COOH	161.46.	165.0

The MS spectrum (Fig. 14) displayed the molecular ion peak at m/e (%rel int.) 302 (19.86, M^+) and base peak at 301 (100.0,M^+-1). This pattern was a characteristic of carboxylic acid. The signal at 287 (21.74, M^+-15) could account for the loss of one methyl group from gem-dimethyl group (302-CH_3) and it also exhibited the dominant fragmentation ion peaks at 258 (3.91,M^+-CO_2), 243 (7.20,287-CO_2), 217 (6.54, 243-C_2H_2) and 213 (10.10, 243-CH_2O). The possible mass fragmentation pattern is given in Scheme 3.3.

Scheme 3.3 The possible mass fragmentation pattern of Compound (3)



The spectral evidences, some of chemical reactions and physical properties of this compound confirmed that this compound was perforatic acid found recently from the root of this plant (*Harrisonia perforata* Merr.) by two Chinese scientists in 1984 [11]. The structure of this compound was shown below:



Compound (3)

3.1.4. Compound (4)

Compound (4) was isolated from the hexane parts of both silica gel and aluminium oxide columns. After recrystallization from methanol, white amorphous product, m.p. 260 C (dec.), Rf value 0.28 (using ethanol as developing solvent and silica gel TLC platr was used) was obtained. The color tests of this compound indicated the presence of a steroidal part, unsaturated part and also a carbohydrate moiety, since it gave a deep green color with Liebermann-Burchard's reagent, decolorized Br_2 in CCl_4 reagent and gave positive result to Molisch's reagents, respectively [36,42-43].

The IR spectrum (Fig. 15) which was assigned in Table 3.8, strongly pointed out an O-H stretching vibration at 3420 cm^{-1}. The C-O stretching vibration of glycosidic linkage was observed at 1080-1030 cm^{-1} and geminal dimethyl group were observed at 1370 cm^{-1}.

Table 3.8 The IR absorption band assignments of Compound (4)

Freguency (cm^{-1})	Band type	Tentative assignment
3600-3400	b	O-H stretching vibration
2950-2870	s	C-H stretching of $-CH_2-$ and $-CH_3$
1640	w	C=C stretching vibration
1470	m	C-H asym. bending of $-CH_2-$ and $-CH_3$
1370	m	C-H bending of gem-dimethyl
1080-1030	s	C-O stretching vibration of glycosidic linkage

The ^1H NMR ($DMSO+CDCl_3$) spectrum (Fig. 16) revealed signals around 0.68-2.20 ppm. typical for steroid moiety. The proton signal at 5.36 ppm. should be olefinic protons. The rest of the signals between 4.79-3.73 ppm. were assigned for the protons on the sugar moiety. The doublet signal at 4.18 ppm. (J=8.00 Hz) which was attributed to an anomeric proton was significant to state the presence of glycosidic linkage. This information was consisted to the IR spectrum and chemical reaction tests that this compound should be composed of steroidal part adjacent to a sugar moiety at C-3 position with glycosidic linkage [52].

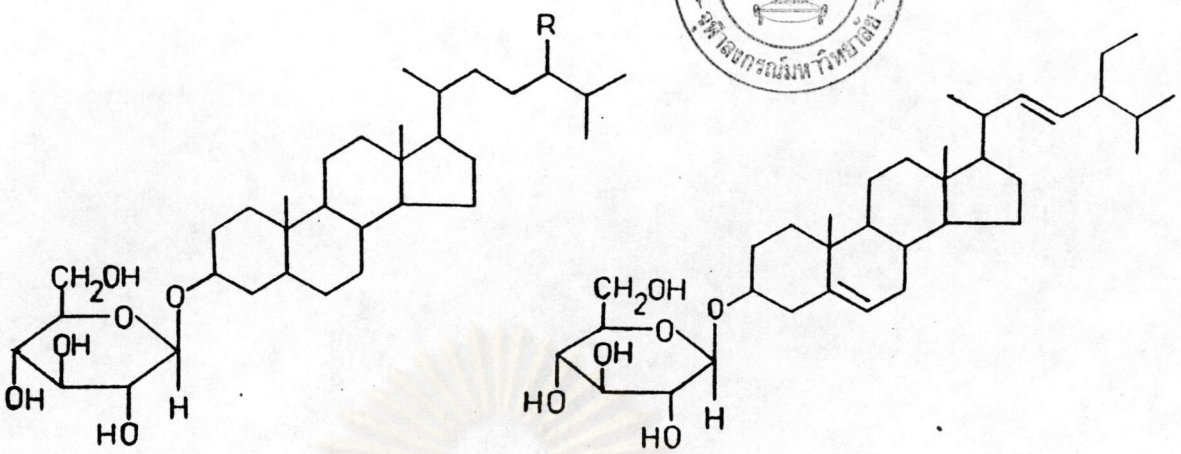
The ^{13}C NMR spectrum (DMSO+$CDCl_3$) (Fig. 17) showed two olefinic carbon signals at 140.63 ppm., 121.68 ppm. and other six carbon signals belonging to the sugar molecule were observed at 101.30-62.50 ppm. Some parts of the carbon signals of this compound were closely resemble to steroidal compounds.

To settle the structure of this compound , the acid hydrolysis reaction of Compound (4) with 10% hydrochloric acid (HCl) in ethanol was conducted and gave aglycone part and a glycone fraction which were assigned as Compound (4a) and Compound (4b), respectively.

The GLC analysis and TLC of Compound (4a) was similar to those of β-sitosterol, stigmasterol and chloresterol.

Compound (4b) was examined by various way such as PC, TLC, HPLC and it was found that it corresponded to D-glucose. Beside using chromatographic method, the acetyl derivative of this compound was prepared and found that its melting point was closed to that of the reported D-glucopentaacetate (112.0 C) [53].

The information above demonstrated that Compound (4) was a mixture of chloresteryl-3-O-glucopyranoside, β-sitosteryl-3-O-glucopyranoside and stigmasteryl-3-O-glucopyranoside. The structure of these compounds are given as follow:



R=H chloresteryl-3-O-glucopyranoside stigmasterol-3-O-glucopyranoside

R=Et β-sitosteryl-3-O-glucopyranoside

Compound (4)

3.1.5. Compound (5)

Compound (5) was collected from the hexane parts using aluminium oxide column and purified by recrystallization from hexane to yield white feather product with wide melting point range 191.0-210.0 °C. Therefore, a purification of Compound (5) was performed by preparing its acetyl derivative. Compound (5) acetate had m.p. 216.5-218.5 °C Rf value 0.70 (solvent 50% chloroform-*n*-hexane and silica gel TLC plate was used). After Compound (5) acetate was hydrolysed by 10% potassium hydroxide (KOH) in ethanol and was worked up in usual manner, the pure Compound (5) was collected as white needle crystals, m.p. 214.0-215.0 °C, Rf value 0.35 using chloroform as a developing solvent (silica gel TLC plate was used). Compound (5) gave a purple color with Liebermann-Burchard's and decolorized Br_2 in CCl_4 reagents. This information suggested that Compound (5) had an unsaturated triterpenoid [36,42-43].

The IR spectrum (Fig. 28) was assigned as in Table 3.9. It showed characteristic absorption bands of secondary alcohol at 3400-3150 cm^{-1} and 1040 cm^{-1} and additional bands of a vinylidine group (CH_3-C=CH_2) at 1370 and 1150 cm^{-1}.

The UV spectrum (Fig. 31) gave the λ_{max} at 205 nm. ($\log \varepsilon = 3.64$) this data showed a non-conjugated system in this compound [54].

Table 3.9 The IR absorption band assignments of Compound (5)

Frequency (cm^{-1})	Band type	Tentative assignment
3400-3200	b	O-H stretching vibration
3080	w	C-H stretching vibration of asym. $R_1R_2C=CH_2$
2980-2870	s	C-H stretching vibration of CH_3, $-CH_2-$
1650	m	non-conjugated C=C stretching vibration
1480-1430	s	C-H bending vibration of $-CH_2-$, CH_3
1370	s	C-H bending vibration of gem-dimethyl
1150	m	skeleton vibration of $(CH_3)_2$-C with no free hydrogen atom on the central carbon

The ^{1}H NMR ($CDCl_3$) spectrum (Fig.29) gave information in good agreement with the IR spectrum information. It exhibited the singlet signals of six methyl proton at chemical shifts : 0.75, 0.80, 0.84, 0.94, 0.96 and 1.05 ppm. (3H each), the broad singlet of vinylic methyl proton at 1.65 ppm. (3H), the broad multiplet of a methine proton attached to a carbon atom bearing a hydroxyl group (-CH-OH) at 3.16 ppm. (1H) and two signals in olefinic region which should be a terminal methylene proton at 4.65 and 4.54 ppm. (2H, J = 9.00 Hz). The ^{1}H NMR chemical shifts of Compound (5) and Compound (5) acetate are presented in Table 3.10.

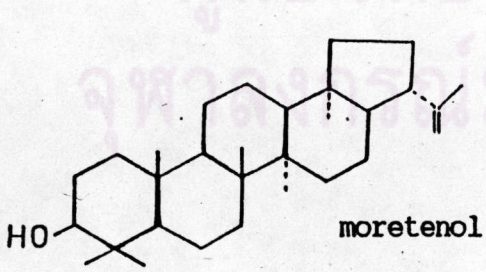
Table 3.10 The ^{1}H NMR chemical shift assignments of Compound (5) and Compound (5) acetate.

Compound	chemical shift (ppm.)			
	olefinic protons	carbinol proton	vinylic methyl protons	methyl protons
Compound (5) acetate (Fig. 24)	4.65,4.52 (2H,J= 10.24 Hz	4.40 (1H,t J=9.04 Hz)	1.66 (3H , s)	0.76 ,0.95 ,1.03 (3H each) , 0.83 (6H)
Compound (5) (Fig. 29)	4.65,4.54 (2H,J=9.0 Hz)	3.14 (1H,t J=7.83 Hz)	1.67 (3H , s)	0.75 ,0.80 ,0.84 0.94 ,0.96 ,1.05 (3H each)
lupeol	4.68,4.78 (2H,J=7.0 Hz)	3.13	1.70	0.78(3H),0.80(3H) 0.84(3H),0.98(6H) 1.05(3H)

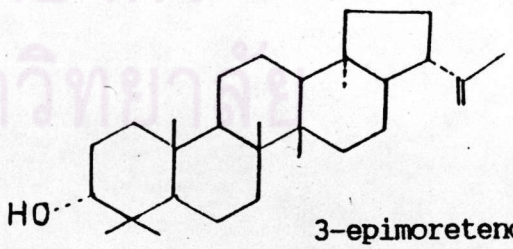
The ^{13}C NMR ($CDCl_3$) spectrum (Fig. 30a) exhibited the olefinic carbon signals at 150.55 and 108.89 ppm.. In ^{13}C NMR DEPT 90 and DEPT 135 spectra (Fig. 30b, 30c) showed signals that corresponded to authentic lupeol.

The mass spectrum (Fig. 32) displayed the molecular ion peak (M^+) at (% rel int.) 426.0 (75.96) (calcd. for $C_{30}H_{50}O$: MW 426.39) with other abundant fragmentation ions at m/e 383.0(5.99), 207.0 (79.54), 189.0 (88.55) and 95.0 (100.00). This peak series pattern implied a triterpenoid compound which belongs to lupane series, i.e., the fragmentation ion peak at m/e 383.0 was due to the loss of isopropynyl group (CH_3-C=CH_2) which was the most important characteristic of triterpenoid compounds [55]. The presence of the ion at m/e 207.0 indicated that the hydroxy group ought to locate at C-3 position [56]. The possible mass fragmentation pattern of Compound (5) is presented in scheme 3.4.

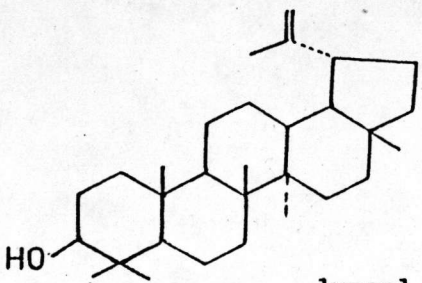
The above information obviously proved that Compound (5) possed a lupane pentacyclic triterpenoidal skeleton with one hydroxy group at C-3 position and one vinylidine moiety, probably in ring E together with six methyl groups. Based on a literature search its sharp melting point, Compound (5) was to be likely one of the following compounds.



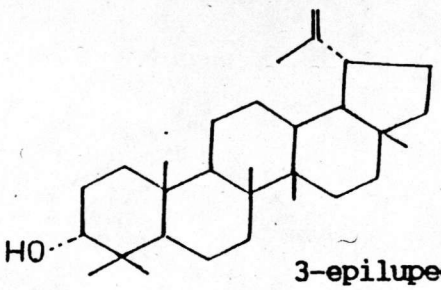
moretenol



3-epimoretenol



lupeol



3-epilupeol

The melting point of Compound (5) could be compared to those of possible compounds, moretenol, 3-epimoretenol, lupeol and 3-epilupeol as given in Table 3.11.

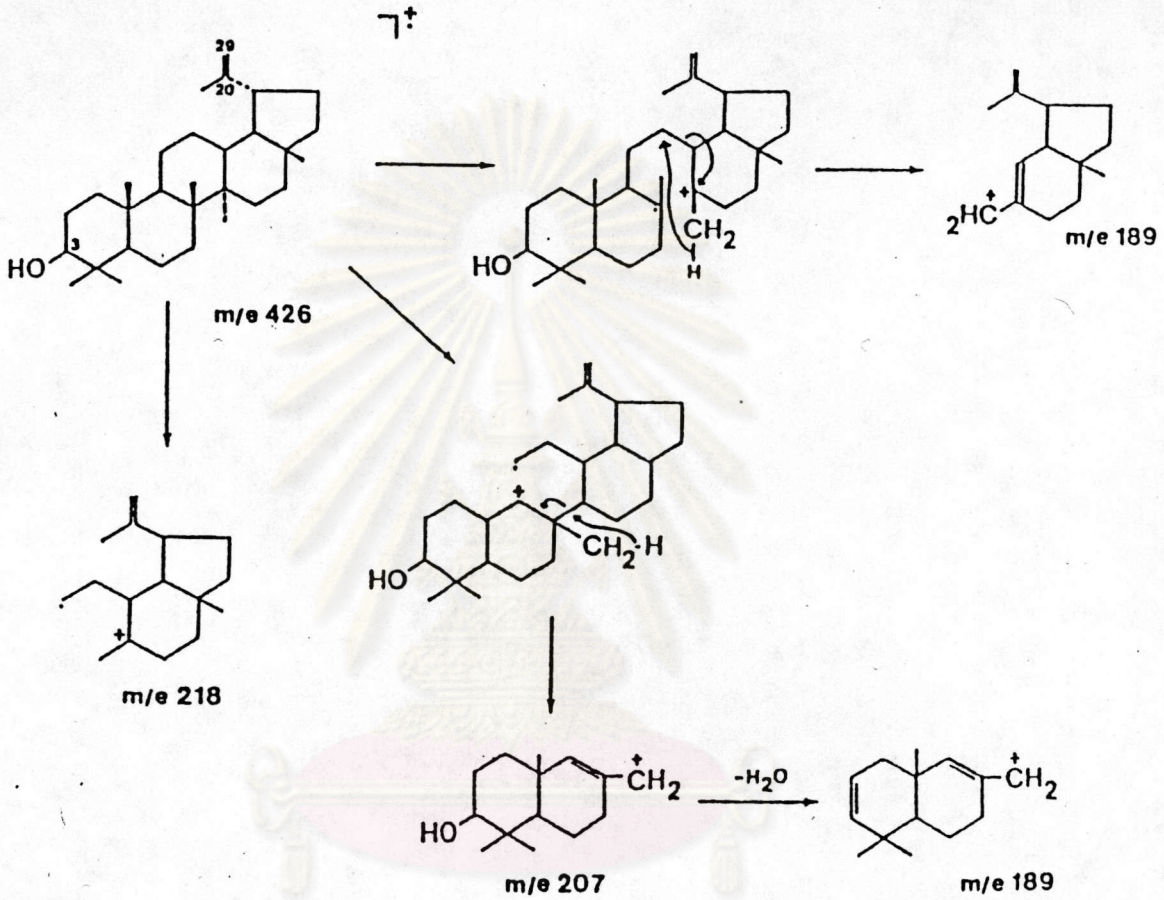
Table 3.11 The comparison data among moretenol, 3-epimoretenol, lupeol, 3-epilupeol and Compound (5)

Compound	melting point (°C)		Ref.
	alcohol	acetate	
moretenol	236	283-285	57,58
3-epimoretenol	223	233-234	58
lupeol	215-216	217-218	57
3-epilupeol	202	-	59
authentic lupeol	214.0-215.0	216.0-218.0	-
Compound (5)	214.0-215.0	216.5-218.5	-

From Table 3.11, it could be seen that Compound (5) was closely to be lupeol rather than other triterpenoids.

To confirm this observation, TLC and GLC analysis of Compound (5) and Compound (5) acetate were carried out by comparing with the authentic compounds. The results of this examination were of no doubt to state that Compound (5) and Compound (5) acetate were lupeol and lupeolacetate, respectively.

Scheme 3.4 The possible mass fragmentation pattern of Compound (5)



3.1.6 Compound (6)

Compound (6) was obtained from the chloroform parts after purification by recrystallization to give white solid, m.p. 68.5-69.5 °C, Rf value 0.67 (4:6 *n*-hexane-chloroform when silica gel TLC plate was used). It was soluble in hot *n*-hexane, chloroform, ether, alcohol and slightly soluble in water. The alkali test revealed that this compound was coumarin.

The IR spectrum (Fig. 34) gave the absorption bands of aromaticity at 3050 cm^{-1} and 1600-1400 cm^{-1}. The absorption peak at 1700 cm^{-1} might be due to the C=O of lactone that conjugated with double bond. The characteristic band for the conjugated double bond appeared at 1460-1625 cm^{-1} [60] and so on aromatic absorption bands were found in their usual regions. The IR absorption band assignments of this compound are presented in Table 3.12 .

The ^{1}H NMR ($CDCl_3$) spectrum (Fig. 35) showed a pair of doublets, J=9.5 Hz at 7.12 (d, 1H) and 6.43 (d, 1H) which strongly indicated a coumarin unsubstituted in pyrone ring. These characteristic signals arised from H-4 (the hydrogen attached to C-4) and H-3 proton respectively [46]. The signals around 7.53 to 7.26 ppm. should be the chemical shifts of aromatic protons.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig. 36) displayed the signal at 160.66 ppm. which was corresponded to carbonyl carbon of lactone. The signals at 153.94 ppm. and 118.74 ppm. could be assign for quaternary carbons by ^{13}C NMR spectrum from DEPT 135 (Fig. 36 C) did not reveal all of this signals. The signals at 143.38 and 116.55 ppm. were assigned to α,β-unsaturated carbonyl carbon.

The signals at 131.73, 127.80, 124.34 and 116.74 ppm. ought to be aromatic carbons.

The tentative ^{13}C NMR chemical shift assignments of Compound (6) as compared with unsubstituted coumarin were shown in Table 3.13.

Table 3.12 The IR absorption band assignments of Compound (6)

Frequency (cm^{-1})	Band type	Tentative assignments
3050	s	C-H stretching vibration of aromatic
1750-1720	s	characteristic for α-pyrone.
1700	s	C=O stretching of α,β-unsaturated lactone
1640-1625	s	C=C stretching of conjugated double bond.
1630-1500	s	C=C stretching of aromatic.
890-870	s	C-H out of plane bending of furan ring

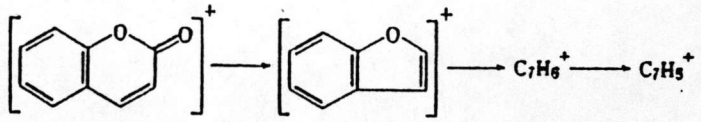
The MS spectrum (Fig. 37) displayed the molecular ion peak at m/e (% rel int.) 146.0 (100.0) which agrees with the molecular formular $C_9H_6O_2$ (proposed from elemental analysis). The fragmentation ion peaks at m/e 118 indicated for lossing 28 mass units from molecular ion. The latter ion occured from lossing carbonyl (CO) from molecular ion directly. The resulting ion has been postulated as the structure of the molecular ion of benzofuran and then benzofuran

ion decomposes further by consecutive loss of CO as showed by the metastable transition $118^+ \longrightarrow 90^+ + 28$ to give the strong peak at m/e 90 corresponding to the ion $C_7H_6^+$ of uncertain structure and a loss of a hydrogen atom from the ion of mass 90 (metastable ion due to $90^+ \longrightarrow 89^+ + 1$) [46,61]. The possible mass fragmentation pattern of this compound was shown in Scheme 3.5.

Table 3.13 The ^{13}C NMR chemical shift tentative assignments of Compound (6) compare with unsubstituted coumarin

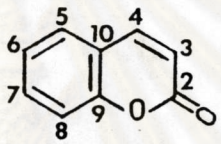
Carbon Position	chemical shift (ppm.)	
	Unsubstituted coumarin	Compound (6)
2	160.3	160.7
3	116.5	116.6
4	143.5	143.4
5	128.0	127.8
6	124.4	124.3
8	116.5	116.7
9	154.0	153.9
10	118.8	118.7

Scheme 3.5



M^+ 146 m/e 118 m/e 90 m/e 89

According to all spectral evidences and chemical reactions it was clearly proved that Compound (6) was unsubstituted coumarin. The structure of this compound was shown below :



Compound (6)

3.1.7 Compound (7)

Compound (7) was obtained from the chloroform parts. After purification by recrystallization to give yellow solid, it had Rf value 0.67 (solvent : 4:6 *n*-hexane :chloroform and silica gel TLC plate was used) and m.p. 68.5-69.5 °C. According to chemical reactions it revealed that this compound is not composed of steroidal and triterpenoidal structures. The alkali test demonstrated that this compound was coumarin.

The IR spectrum (fig. 38) revealed the characteristic absorption bands of alcohol at 3500-3100, 1220 and 660 cm^{-1}. At 1710, 1200 and 1150 cm^{-1} correspond to lactone. The absorption bands of aromatic appeared at 1620-1470 cm^{-1} and ether appeared at 1445, 1120 and 1020 cm^{-1}. The IR absorption bands of

Compound (7) were shown in Table 3.14.

Table 3.14 The IR absorption band assignments of Compound (7)

Frequency (cm^{-1})	Band type	Tentative assignment
3500-3100	s	O-H stretching vibration of alcohol
3060	s	C-H stretching vibration of alkene and aromatic
2000-1650	summation	C-H bending of aromatic
1710	b	C=O stretching vibration of lactone
1620-1490	s	C=C stretching vibration of aromatic
1445	s	C-H bending of ether
1395	m	C-H bending of aromatic
1360	s	O-H in plane bending
1260,1120,1020	s	C-O-C stretching of ether
1200 , 1150	s	C-O stretching of lactone
930 , 850	m	C-H bending of aromatic

The ^1H NMR ($CDCl_3$) spectrum (Fig. 39) reveals a pair of doublet, at 7.96 ppm. (d, 1H) and 6.21 ppm.(d, 1H) (J = 9.5 Hz), strongly indicated for a coumarin unsubstituted in pyrone ring. These characteristic signals arised from H-4 and H-3 protons, respectively [46] and the signal at 6.44 ppm. should be

chemical of hydroxyl proton. The signal at 6.40 ppm. was the H-8 signal and the signal of methoxy proton found at 3.89-3.92 ppm.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig. 40) displayed for eleven carbon signal. The signals at 56.18 ppm. and 61.34 ppm. corresponded to methoxy protons. In addition to being a substitued coumarin, positionsso for each carbon signal could be calculated from substituent chemical shift (SCS) of unsubstituted coumarin.

The C-13 NMR chemical shifts calculation of substituted coumarin

The effect of hydroxyl group and methoxy group when present in the carbocyclic ring, was quite characteristic. The newly formed quaternary carbon moves approximately 30, 33 ppm., respectively downfield from the value observed in coumarin itself. Carbons ortho and para to the substituent both move upfield by 11-15 ppm. and 7-10 ppm., respectively, while those which are meta appear a little affected, but move consistently downfield by 0.3-2.3 ppm. [62,63].

Example The calculation of ^{13}C NMR chemical shift of C-5, C-6, C-7 and C-9.

$$\delta_{C-5} = \delta_{C-5\ (unsubstituted)} + SCS_{5-OH} + SCS_{ortho,5-OH} + SCS_{meta,7-OMe}$$

$$= 128.0 + 30 - 13 + 1.3$$

$$= 146.3 \text{ ppm.}$$

$$\delta_{C-6} = \delta_{C-5\ (unsubstituted)} + SCS_{6-OMe} + SCS_{ortho,5-OH} + SCS_{ortho,7-OMe}$$

$$= 124.4 + 33 - 13 - 13$$

$$= 131.4 \text{ ppm.}$$

$$\delta_{C-7} = \delta_{C-7\ (unsubstituted)} + SCS_{7-OMe} + SCS_{ortho,6-OMe} + SCS_{meta,5-OH}$$

$$= 131.7 + 33 - 13 + 1$$

$$= 152.7 \text{ ppm.}$$

$$\delta_{C-9} = \delta_{C-9\ (unsubstituted)} + SCS_{meta,5-OH} = SCS_{para,6-OMe} + SCS_{meta,7-OMe}$$

$$= 154.0 + 1.9 + 8.5 + 1$$

$$= 159.6 \text{ ppm.}$$

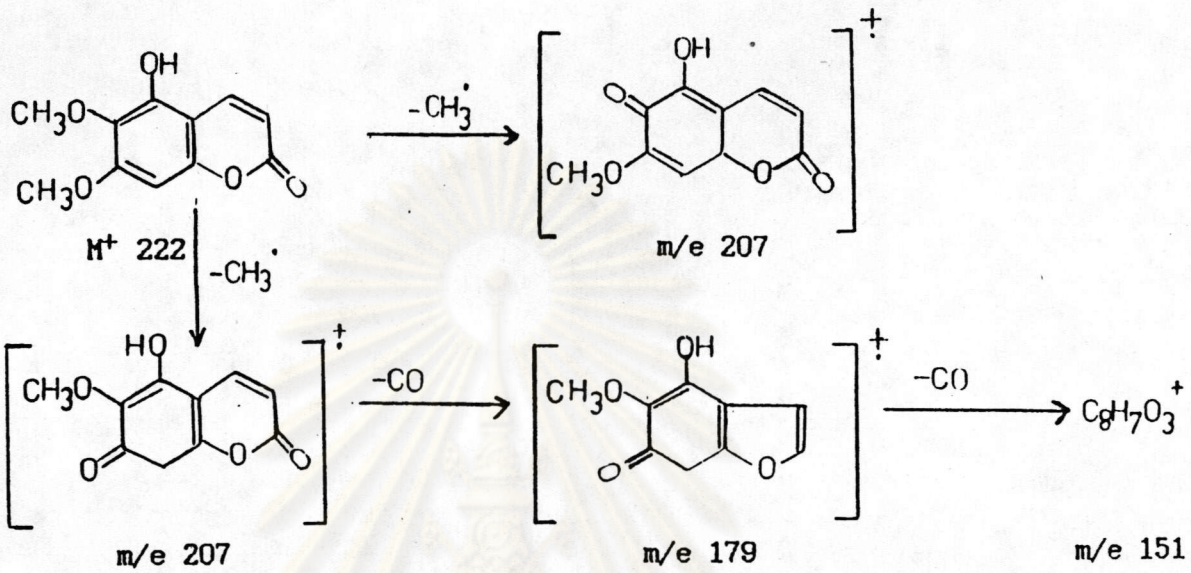
The MS spectrum (Fig. 41) displayed the molecular ion peak at m/e 222 (79.9) and the other fragmentation ions at m/e 207 (100.0), 179 (15.16) and 151 (29.11).

The literature search of methoxy coumarin [64,65] fragmentation indicated that the methyl radical and two groups of carbonyl were removed from the remainder ion to give ion at m/e 207, 179 and 151. By the way, loss of methyl radical gives quinonoid structure which was very stable. Therefore, after this compound loss methyl radical at C-7 (m/e 207) to give p-quinonoid structure, this peak at m/e 207 was also a base peak. The possible mass fragmentation patterns of this compound were presented Scheme 3.6.

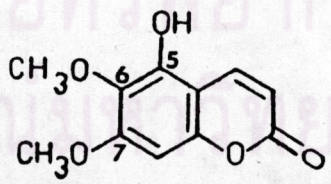
Table 3.15 The ^{13}C NMR chemical shifts of unsubstituted coumarin, the observed chemical shifts of Compound (7) and the calculated chemical shifts of Compound (7)

carbon position	Chemical shift (ppm.)		
	observed (cpd 7)	calculated (cpd 7)	unsub.coumarin
2	161.47	160.3	160.3
3	111.68	116.5	116.5
4	138.61	143.5	143.5
5	147.66	146.3	128.0
6	131.55	131.4	124.4
7	151.12	152.7	131.7
8	92.31	94.2	116.5
9	155.61	159.6	154.0
10	102.53	100.2	118.8

Scheme 3.6 The possible mass fragmentation pattern of Compound (7)



The spectral data, chemical reaction and physical properties demonstrated that Compound (7) was 5-hydroxy-6,7- dimethoxy coumarin or tomentin that found in *Prenus tomentosa* [66]. The structure of this compound was shown below :



Compound (7)

3.1.8 Compound (8)

Compound (8) was eluted from the chloroform parts which was separated on the silica gel column. A white amorphous solid was obtained by recrystallization. The collected compound showed a single spot on silica TLC plate with Rf value 0.57 by using chloroform as a developing solvent. This compound gave negative results to Liebermann-Burchard's reagent and Br_2 in CCl_4 reagents. This result suggested that there were neither steroidal, triterpenoidal nor unsaturation part in molecule [36,42-43].

The IR spectrum (Fig. 42) displayed the absorption peak at 3350 cm^{-1} indicated that this compound contain hydroxyl group as a functional group. This information could be confirm by the absence of this peak when this compound was acetylated (Fig. 48). The characteristic peak at 1050 cm^{-1} was important to state C-O stretching vibration of primary alcohol. The absorption band at 1460 cm^{-1} to show $-CH_2-$ and $-CH_3$ bending. The IR absorption bands of this compound were shown in Table 3.16.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig. 44) displayed the signal of one carbon at 63.10 ppm. which was the carbon adjacent to hydroxyl group. The signal at chemical shift around 32.85 to 22.75 ppm. refered to the chemical shift of methylene carbons. The rest signal at 14.0 ppm. was assigned for the methyl carbon [67].

Table 3.16 The IR absorption band assignments of Compound (8)

Frequency (cm^{-1})	Band type	Tentative assignment
3350	b,s	O-H stretching vibration
2940,2860	s	C-H stretching vibration of $-CH_3$ $-CH_2-$
1490,1480	m	C-H bending vibration of $-CH_3$,$-CH_2-$
1050	m	C-O stretching vibration of primary alcohol
730,720	w	C-H rocking mode of $-CH_2-$ (for C > 4)

The ^1H NMR (Fig. 43) exhibited the important peak that was triplet signal at 3.67 ppm. (2H) which should be the signal of the proton on carbon attaching to heteroatom. In IR spectrum showed absorption band that belonging to oxygen atom, so this heteroatom should be an oxygen atom. The presence of high intensity singlet signal at 1.27 ppm. revealed that there were several interlinking of methylene groups in the molecule of this compound. The singlet signal at 0.90 ppm. (3H) was corresponded to the methyl proton [68].

The molecular formular of Compound (8) was proposed to be $C_{34}H_{70}O$. This formular was supported by the mass spectrum data. In mass spectrum (Fig. 45) of saturated aliphatic alcohol which

contain many carbon atoms, the molecular ion peak, M^+ is usually very weak or missing. However, it always shows the prominent peak corresponded to the successive loss of one molecule of water from the molecular ion peak (M^+-18) together with the elimination of an olefin [68-69]. Therefore, the fragmentation pattern of aliphatic primary alcohol is M^+ (sometime missing), M^+-H_2O, M^+-H_2O-olefin, i.e., (M^+-18) (M^+-46), (M^+-74) and so on. The mass spectrum of Compound (8), the molecular ion peak was missing and there was the prominent one at m/e (% rel int.) 476.0 (3.50) which should be loss of one molecule of water (M^+-18) peak. The other peaks were corresponded to the fragmentation pattern of saturated primary alcohol, there were at 448.0 (12.85, $M^+-H_2O-(CH_2)_2$, M^+-46), 420.0 (14.25, 448.0-$(CH_2)_2$), 392.0 (4.85, 420.0-$(CH_2)_2$), 364.0 (2.15, 392.0-$(CH_2)_2$), 336.0 (2.00 364.0-$(CH_2)_2$), 167.0 (11.50, 336.0-$(CH_2)_{11}CH_3$) and 97.0 (167.0-$(CH_2)_5$) This information revealed that the molecular structure of this compound was composed of one methyl group, thirty three of methylene group and a hydroxyl functional group. Addition to these information, Compound (8) should be a saturated long chain primary alcohol.

By the way, the GLC data revealed that compound (8) is a mixture of 5 saturated long chain aliphatic primary alcohol. The plotted standard correlation curve between logarithm of retention time and the number of carbons in the authentic primary long chain alcohol samples* [70]. The GLC analysis result and the standard correlation

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*Standard samples are tetradecanol ($C_{14}H_{29}OH$) , hexadecanol ($C_{16}H_{33}OH$) octadecanol ($C_{18}H_{37}OH$) , icosanol ($C_{20}H_{41}OH$) and doicosanol ($C_{22}H_{45}OH$).

curve revealed 5 peaks on gas chromatogram (Fig. 46 and Fig. 47) at retention time 9.60, 11.76, 14.78, 18.29 and 22.96 min., respectively which were corresponded to number of carbon 31, 32, 33, 34 and 35 respectively. The substances at retention time 18.39 was the major components in this mixture.

Compound (8) was infact a mixture of 5 saturated long chain aliphatic primary alcohol; hentriacontanol ($C_{31}H_{63}OH$), dotriacontanol ($C_{32}H_{65}OH$), tritriacontanol ($C_{33}H_{67}OH$), tetratriacontanol ($C_{34}H_{69}OH$) and pentatriacontanol ($C_{35}H_{71}OH$) respectively. Tetratriacontanol ($C_{34}H_{69}OH$) was the major components in this mixture. The structure of Compound (8) was shown below :

$$CH_3(CH_2)_nCH_2OH \qquad n=29,30,...,33$$

Compound (8)

3.1.9 Structural Elucidation Compound (9)

Compound (9) was isolated from the methanol parts and purified by recrystallization from the mixture of chloroform and methanol to yield yellow amorphous compound, decomposed at 250 °C. This compound decolorized Br_2 in CCl_4 and gave negative result to Liebermann-Burchard's reagent. This results revealed that the compound had unsaturated part but not composed of steroid and triterpenoid part, respectively.

The IR spectrum (Fig. 49) which was assigned in Table 3.17 revealed that the characteristic of hydroxyl group of

alcohol displayed the signal at 3600-3200 cm^{-1}. Aromatic streching vibration at 3040 cm^{-1}. The conjugated carbonyl appeared at 1670 cm^{-1}. The signals at 1390 and 1380 cm^{-1} were assigned for geminal dimethyl group together with C-O streching of primary alcohol at 1060 cm^{-1}.

Table 3.17 The IR absorption band assignments of Compound (9)

Frequency (cm^{-1})	Band type	Tentative assignment
3600-3200	b	O-H streching vibration of alcohol
3040	s	C-H streching vibration of aromatic and alkene
1670	s	C=O streching vibration of α,β-unsaturated carbonyl
1620	s	C=C streching vibration
1600-1400	s	C=C streching vibration of aromatic
1390,1380	s	geminal dimethyl
1060	s	C-O streching vibration of primary alcohol

The ^1H NMR ($CDCl_3$) spectrum (Fig. 50) exhibited two doublet signals at 6.44 ppm. and 5.47 ppm. (J = 10.0 Hz) should be olefinic proton (-CH=CH-). The singlet signal of aromatic carbon which adjacent to quarternary carbon showed the signal at 6.25 ppm..

The methoxy proton (3H) showed the singlet signal at 3.85 ppm. The methyl group showed two of singlet signals, the first one (3H) at 2.25 ppm. and the last one (6H) belongs to gem-dimethyl groups at 1.43 ppm.

The ^{13}C NMR ($CDCl_3$) spectrum (Fig. 51) exhibited the carbonyl carbon at 177.54 ppm. The singlet signals at 164.56 ppm., 160.61 ppm. and 157.16 ppm. were assigned for quarternary carbon that adjacent to oxygen atom and the signals at 154.10 ppm., 114.06 ppm., 108.52 ppm. and 102.17 ppm. were assigned for quarternary carbon. The signals at 127.20 ppm. and 115.02 ppm. should be carbon-carbon double bond. The methoxy carbon and geminal dimethyl carbon showed the signals at 56.09 ppm. and 27.76 ppm., respectively. The signals at 30.60 ppm. and 61.10 ppm. belongs to methyl carbon and hydroxy methyl carbon ($-CH_2OH$), respectively.

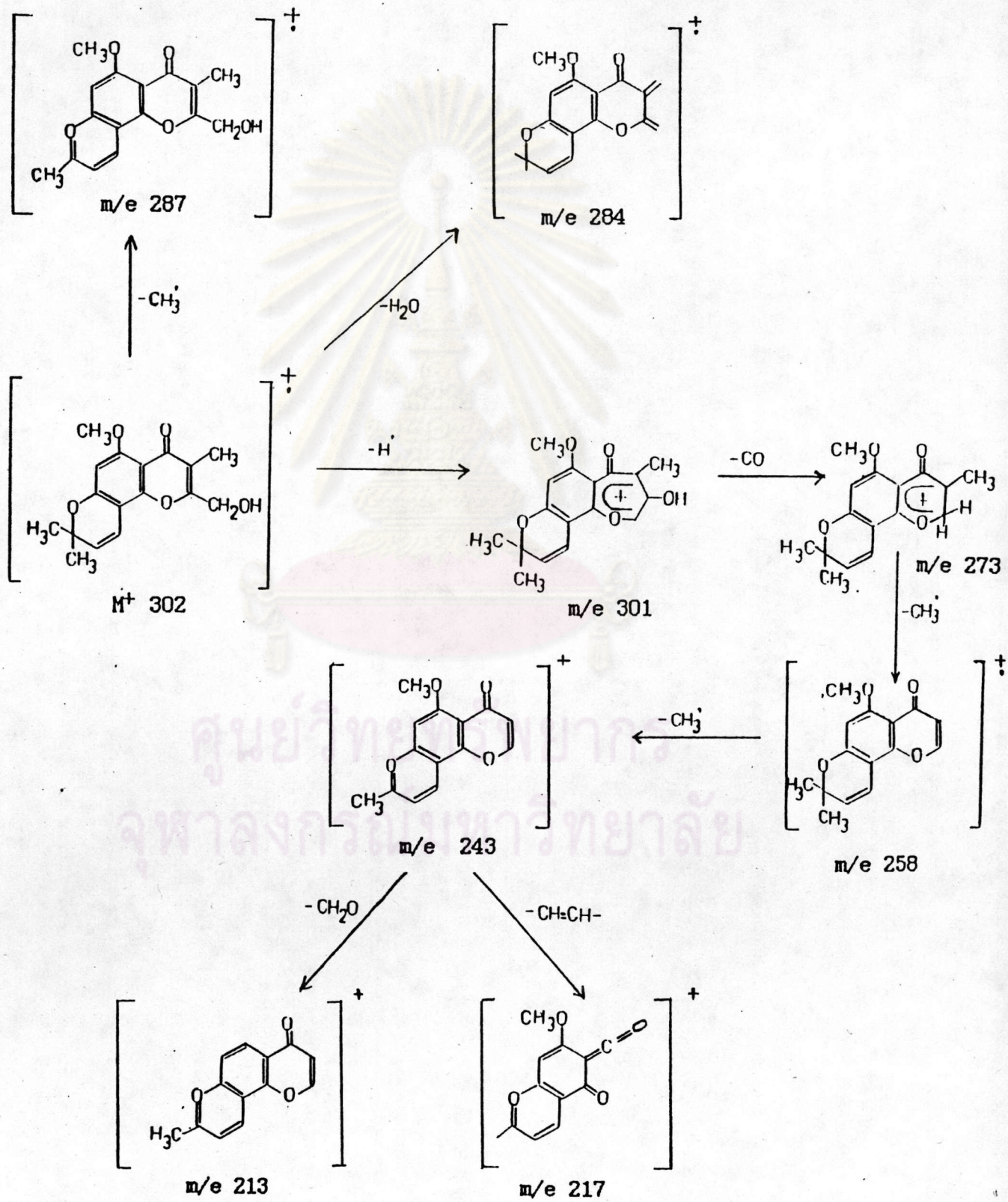
A search through the literature revealed that Compound (9) exhibited the ^{13}C NMR chemical shift closely to 2-hydroxymethylalloptaeroxylin [71]. The comparison between ^{13}C NMR chemical shift of Compound (9) and 2-hydroxymethylalloptaeroxylin could be assigned in Table 3.18.

Table 3.18 The ^{13}C NMR chemical shifts of Compound (9) and 2-methylhydroxyalloptaeroxylin

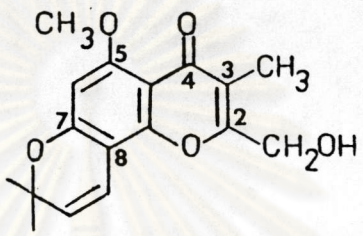
Carbon	Chemical shift (ppm.)	
	Compound (9)	2-methylhydroxy alloptaeroxylin
2	164.56	164.97
3	114.06	109.91
4	177.54	178.03
5	154.10	154.00
6	96.36	96.60
7	160.02	160.61
8	108.52	108.74
9	157.16	157.98
10	102.17	102.48
1'	77.92	78.10
2'	127.20	127.36
3'	115.02	115.10
4'	27.76	28.26
5'	27.76	28.26
$-CH_3$	30.60	-
$-OCH_3$	56.09	56.31
$-CH_2OH$	61.10	61.16

The MS spectrum (Fig. 52) displayed the a very weak molecular ion peak at m/e 302 (M^+) and base peak at m/e 284 (M^+-H_2O,M^+-18) that corresponded to the successive loss of one molecule of water from the molecular ion peak. This peak is most noticeable in spectra of primary alcohol. The signal at m/e 287 (M^+-CH_3) could be assigned for the loss of one methyl radical from the molecular ion peak. The ion m/e 301 (M^+-H) arised by lossing a hydrogen radical. The fragmentation of the ion m/e 273 lost a carbonyl group (CO), as showed by the metastable transition 301 --> 273^+ + 28 and then it lost two methyl radical to give signal at m/e 258 ($273-CH_3$) and 243 ($258-CH_3$), respectively. The fragmentation of ion at m/e 243 lost an ethylene group to give signal at m/e 217. However, the fragmentation at 243 also lost a $-CH_2O$ group to give signal at m/e 213. The possible mass fragmentation pattern was shown in Scheme 3.7.

Scheme 3.7 The possible mass fragmentation pattern of Compound (9)



According to spectrum evidances and some of chemical reactions it is confirmed that this compound is 2-hydroxymethyl-3-methylalloptaeroxylin ($C_{17}H_{18}O_5$). The literature search revealed that it is a novel Compound. The structure of this compound was shown below :



Compound (9)

3.2 Biological Activities and Utilizations of the Isolated Compounds

The reported biological activities and utilization of the isolated compounds were presented as follow. However, the study of biological activities and utilizations was not performed in this case study.

3.2.1 Compound (1)

Compound (1) was identified as a mixture of β-sitosterol, stigmasterol and campesterol. The co-occurance of these three steroids is widely distributed in the plant kingdom. The plant steroids were well known to be used as precursor for preparing steroid hormones. For example, stigmasterol has been reported to be used as precursors for synthesize progesterone hormone [72], β-sitosterol is proposed for treatment of hypercholesterolemia [73]. Moreover, the mixture of steroids displayeds the antifeedant activity [74].

3.2.2 Compound (2)

Compound (2) was elucidated as a heteropeucenin-7-methyl ether. This compound is a major component in roots of *Harrisonia perforata* Merr. However, there has not been reported abou its activities or utilization.

3.2.3 Compound (3)

Compound (3) was elucidated to be a perforatic acid. Literature surveys stated that this compound is an inhibitory action hepatic carcinoma [12].

3.2.4 Compound (4)

Compound (4) was elucidated to be a mixture of β-sitosteryl-3-O-glucopyranoside, stigmasteryl-3-O-glucopyranoside and chloresteryl-3-O-glucopyranoside. Literature surveys stated that it is β-sitosteryl-3-O-glucopyranoside abundant sterylglycoside. It was reported that this compound has effected on vascular permeability, antiulcerrogenic and hemostatic effect [75] and showed growth promoting activity in human [76]. It was also reported that a mixture of sterylglycoside containing β-sitosteryl-3-O-glucopyranoside showed antitumor activity against leukemia [77-78]. Moreover, this compound revealed inflammatory activity and was said to be used as drug carriers.

3.2.5 Compound (5)

Compound (5) was identified as lupeol. This compound is a pentacyclic triterpene belonging to a lupane group. This triterpenoid compound was found to be one of the most widely distributed compound in mangrove plants [79]. Lupeol was an effective compound for rheumatism and urinary infections [80]. It also showed significant antitumor activity in Spraque rats against the

tumor system [81]. Moreover, lupeol had been reported to infect with the fungus *Verticillium* [82]. In recent years, the acetyl derivative of this compound (lupeolacetate) was found to be used as the inhibitor of incidence of stress-induced ulcers and decreased the incidence of gastric ulceration induced by pyloric ligation [83].

3.2.6 Compound (6)

Compound (6) was elucidated as an unsubstituted coumarin. This compound is widely distributed in the leaves and branch of the plant extract. Literature surveys stated that this compound was used as a flavoring agent for formulating some drugs. It was stated in the literature that this compound is an inhibitory action of vitamin K and have effect on human vascular system [44].

3.2.7 Compound (7)

Compound (7) was elucidated as a 5-hydroxy-6,7-dimethoxy coumarin. The coumarin are widely distributed in the leaves of the plant extract. The 5,6-dimethoxy coumarin has been reported to be an inhibitor on the growth of the fungus *Phytophthora citrophthora, Verticillium dahliae, Penicillium digitatum, Penicillium italicum, Collectotrichum glocosporioides, Diplodia natalensis and Hendersonula toruloidea* [84]. Similarly, it might be assumed that Compound (7) should have inhibitory effect on the growth of fungus.

3.2.8 Compound (8)

Compound (8) was elucidated as a mixture of saturated long chain aliphatic alcohols (C_{31}-C_{35}). The saturated long chain aliphatic alcohols are widely distributed in the waxy fraction of the plant extract. The triacontanol ($C_{30}H_{61}OH$) had been reported to be used as a plant growth regulator (PGR) [85-86]. In addition, Compound 8 has a number of carbon atoms close to triacontanol so that Compound(8) might be shown the activities closely to triacontanol.

3.2.9 Compound (9)

Compound (9) was separated from the methanol part that was elucidated structure as 2-hydroxymethyl-3-methylalloptaeroxylin. This compound is a novel compound. Literature search stated that alloptaeroxylin is an inhibitory action of hepatic carcinoma [12], antibacterial and antifeedant properties [71]. In addition, Compound (9) is a derivative of alloptaeroxylin so that this compound might be shown the activities closely to alloptaeroxylin.

3.3 The Isolated Compounds from Roots of *Harrisonia perforata* Merr.

Compound (1) : bright white needle crystals, m.p. 128-130 °C, was identified as a mixture of steroids : β-sitosterol, campesterol and stigmasterol.

Compound (2) : bright pale yellow needle crystal, m.p.103.0-104.0 °C, was elucidated as heteropeucenin-7-methylether.

Compound (3) : pale yellow amorphous compound, decomposed at 230 °C, was elucidated as perforatic acid.

Compound (4) : white amorphous compound,decomposed at 260 °C, was identified as a mixture of steroidal glycoside : β-sitosteryl-3-O-glucopyranoside, stigmasteryl-3-O-glucopyranoside and chloresteryl-3-O-glucopyranoside,

Compound (5) : bright white needle, m.p. 213.5-215.0 °C, was elucidated as lupeol.

Compound (6) : white needle solid, m.p. 68.5-69.5 °C, was elucidated as a unsubstituted coumarin.

Compound (7) : pale yellow needle solid, m.p. 183.5-184.0 °C, was elucidated as 5-hydroxy-6,7-dimethoxy-coumarin.

Compound (8) : white amorphous solid, m.p. 84.5-85.5 °C, was elucidated as a mixture of saturated long chain aliphatic alcohol.

Compound (9) : yellow amorphous compound, decomposed at 250 °C, was identified as 2-hydroxymethyl-3-methyl-alloptaeroxylin.

The isolated compounds from roots of *Harrisonia perforata* Merr. were presented in Scheme 3.8

Scheme 3.8 The isolation compounds from roots of *Harrisonia perforata* Merr.