

CHAPTER III

RESULTS AND DISCUSSION

Structural elucidation of the eluted compounds from crude extract of hexane, dichloromethane, ethyl acetate and methanol of woods of *Croton oblongifolius* Roxb. are described in the following sections.

Structural Elucidation of Compound I

Compound I was obtained as a white solid (24.1 mg) and has a melting point of 57-58 °C. This compound was separated by column chromatography of the hexane crude extract (equivalent to 0.13 % wt. by wt. of hexane crude extract). It was soluble in hexane, dichloromethane and chloroform but insoluble in ethyl acetate, methanol and acetone. Its IR spectrum was shown in Fig. 6 and the absorption band assignments was presented in Table 8.

Table 8 The IR Absorption Band Assignments of Compound I

Wavenumber (cm ⁻¹)	Intensity	Vibration
2925, 2860	s, s	C-H stretching vibration of -CH ₂ -, -CH ₃
1470	m	C-H bending vibration of -CH ₂ - and asymmetric bending vibration of -CH ₃
1380	w	C-H symmetric bending vibration of -CH ₃
805	w	C-H rocking vibration
725	w	C-C bending vibration of -(CH ₂) _n - ; n ≥ 4

IR spectrum indicated C-H stretching vibration peaks of aliphatic C-H of alkanes at 2925 and 2860 cm^{-1} . The absorption peaks at 1470 and 1380 cm^{-1} corresponded to C-H bending vibration mode of $-\text{CH}_2-$ and $-\text{CH}_3$ group, absorption peaks at 805 cm^{-1} corresponded to C-H rocking vibration and absorption peak at 725 cm^{-1} indicated one or more saturated long chain of $-(\text{CH}_2)_n$; $n \geq 4$.

By comparison to IR spectrum of standard long chain aliphatic hydrocarbon (22), both spectra show close similarity. Compound I was further analysed by GC technique. The chromatogram appeared as seven peaks at different retention times as shown in Table 9 and Fig. 7.

Comparison to Gas chromatogram of standard long chain aliphatic hydrocarbons ($\text{C}_n\text{H}_{2n+2}$; $n = 24-33$) (Fig. 7) using calibration curve of log retention time with number of carbon (Fig. 8) revealed that Compound I was a mixture of seven long chain aliphatic hydrocarbons ($\text{C}_n\text{H}_{2n+2}$; $n = 27-33$).

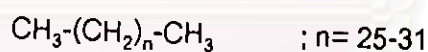
Table 9 The Retention Times of Compound I

Retention time (min)	Log retention time	Number of carbon
6.26	0.80	27
8.01	0.90	28
10.22	1.01	29
13.30	1.12	30
17.12	1.23	31
22.19	1.35	32
28.59	1.46	33

Table 10 Name of Long Chain Aliphatic Hydrocarbons in Compound I

Molecular formula	Molecular weight	Name of substance	% Composition
$C_{27}H_{56}$	380	heptacosane	0.49
$C_{28}H_{58}$	394	octacosane	1.27
$C_{29}H_{60}$	408	nonacosane	39.07
$C_{30}H_{62}$	422	triacontane	4.55
$C_{31}H_{64}$	436	hentriacontane	37.97
$C_{32}H_{66}$	450	dotriacontane	4.81
$C_{33}H_{68}$	464	tritriacontane	11.84

Thus, Compound I was a mixture of heptacosane ($C_{27}H_{56}$), octacosane ($C_{28}H_{58}$), nonacosane ($C_{29}H_{60}$), triacontane ($C_{30}H_{62}$), hentriacontane ($C_{31}H_{64}$), dotriacontane ($C_{32}H_{66}$) and tritriacontane ($C_{33}H_{68}$). Nonacosane and hentriacontane were the major components, 39.07 % and 37.97 %, respectively while heptacosane was the trace component, 0.49 %. The structure of Compound I was shown below :



Structural Elucidation of Compound II

Compound II appeared as white needle like crystals from column chromatography of hexane crude extract and dichloromethane crude extract. It was purified by recrystallization from hexane/chloroform for several times to obtain approximately 998.2 mg (equivalent to 847.2 mg = 4.48 % wt. by wt. of hexane crude extract and 151.0 mg = 0.74 % wt. by wt. of dichloromethane crude extract). The melting point of Compound II was 152-154 °C and the R_f value was 0.55 (silica gel, 5 % MeOH/ $CHCl_3$). This compound was soluble in hot hexane, dichloromethane, chloroform and slightly soluble in ethyl acetate and methanol.

Fig. 9 showed the IR spectrum of Compound II and the absorption bands were assigned as shown in Table 11. The IR spectrum revealed the presence of O-H at 3100-3700 cm^{-1} (very broad), C=C stretching vibration peak at 1635 cm^{-1} , C-O stretching peak at 1262 cm^{-1} and absorption band at 1024-1092 cm^{-1} indicated the C-O bending vibration of R-OH and absorption band of an unsaturated system at 807 cm^{-1} .

Table 11 The IR Absorption Band Assignments of Compound II

Wavenumber (cm^{-1})	Intensity	Vibration
3100-3700	s	O-H stretching vibration of R-OH
2960,2868	m,m	C-H stretching vibration of $-\text{CH}_2-$, $-\text{CH}_3$
1635	m	C=C stretching vibration of alkene
1459	w	C-H bending vibration of $-\text{CH}_2-$ and asymmetric bending vibration of $-\text{CH}_3$
1379	w	C-H symmetric bending vibration of $-\text{CH}_3$
1262	m	C-O stretching vibration of R-OH
1024-1092	m	C-O bending vibration of R-OH
807	m	C-H bending out of plane of trisubstituted vinyl

The $^1\text{H-NMR}$ spectrum (Fig. 10) exhibited the signals of protons attached to sp^3 carbons in $-\text{CH}_3$, $-\text{CH}_2-$ and $-\text{CH}-$ group between δ 0.66-2.26 ppm. The signal at δ 3.50 ppm was a proton attached to a carbon bearing a hydroxy group ($-\text{C}-\text{OH}$). Two signals of olefinic protons (1H each) were observed at δ 5.10 and 5.35 ppm.

The $^{13}\text{C-NMR}$ spectrum (Fig. 11) showed signals of sp^3 carbons between δ 11.85-56.86 ppm. Other signals were detected at δ 71.73 ppm which was the signal of a $-\text{C}-\text{OH}$ group and at δ 121.67, 129.26, 138.31 and 140.75 ppm which were the signals of sp^2 carbons of $-\text{CH}-$, $-\text{CH}-$, $-\text{CH}-$ and $-\text{C}-$, respectively.

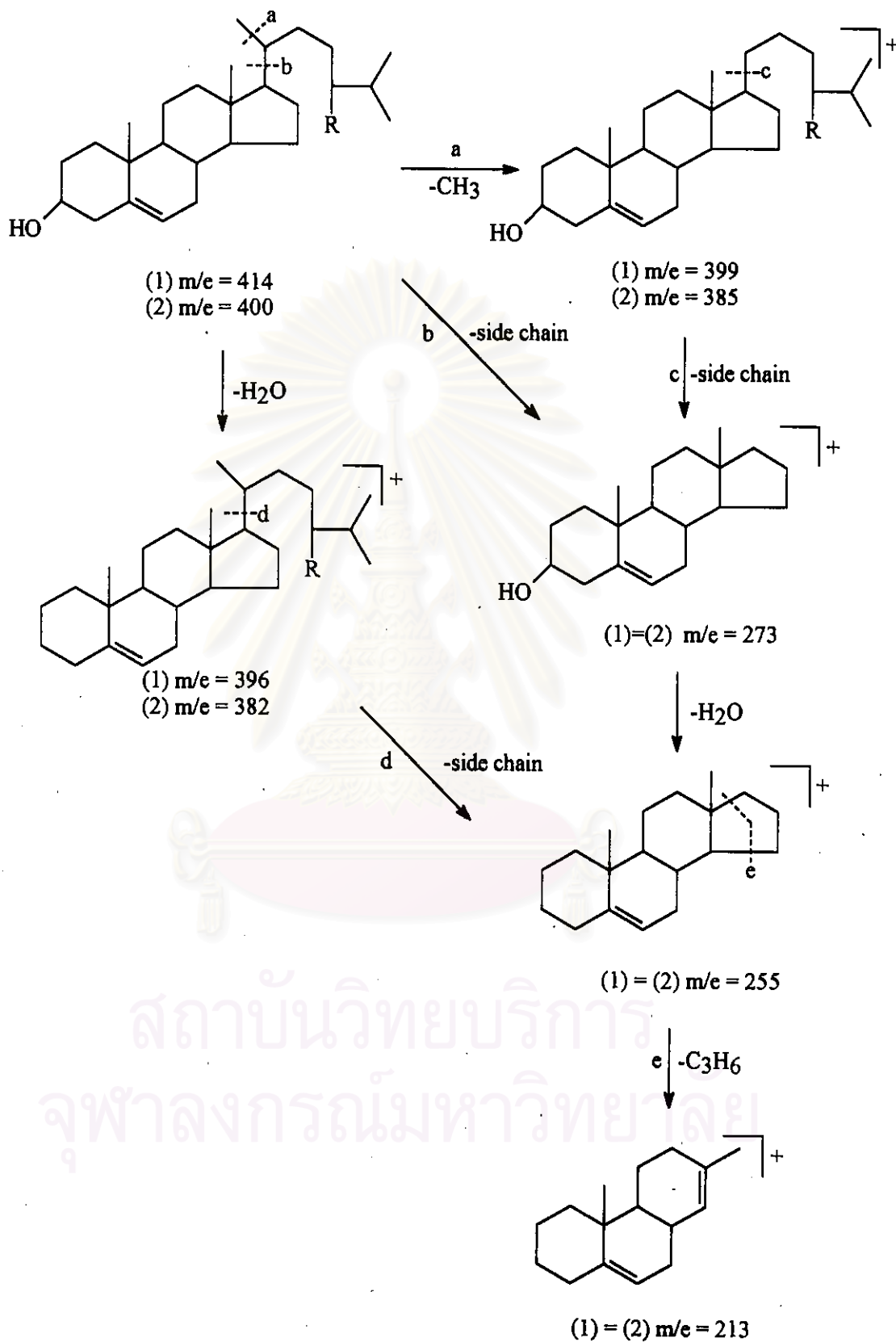
The information obtained from the IR spectrum, $^1\text{H-NMR}$ spectrum and $^{13}\text{C-NMR}$ spectrum suggested this compound should be a steroid.

Compound II was analysed by GC technique, its chromatogram was shown in Fig. 12 and was compared to mixture of three standard steroids including campesterol, stigmasterol and β -sitosterol. The retention times of the standard steroids were shown in Fig. 12. Compound II was thus shown to be a mixture of campesterol, stigmasterol and β -sitosterol. Table 12 showed the retention times of Compound II and standard steroids.

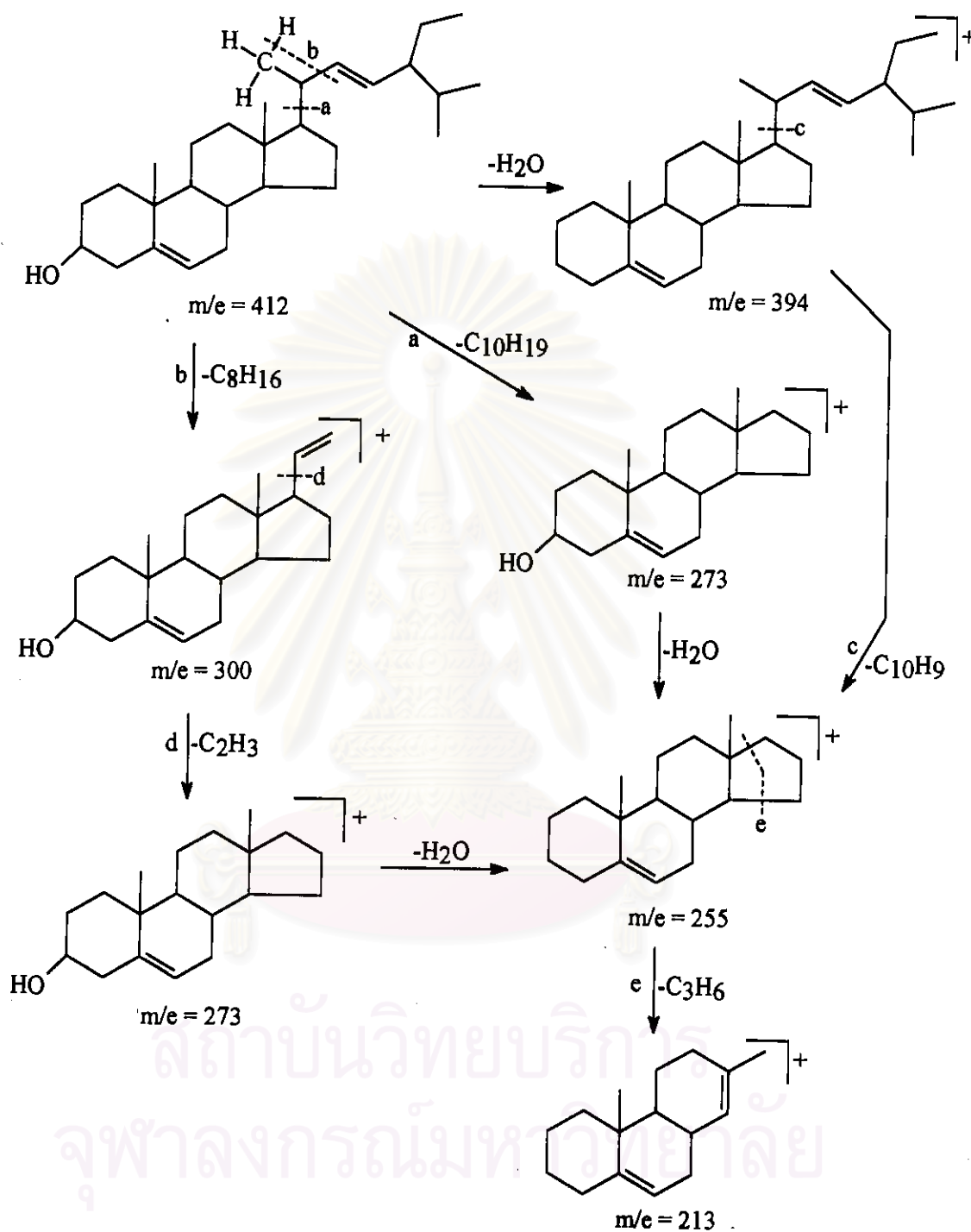
Table 12 The Retention Times of Compound II and Standard Steroids.

Compound	Retention time	% composition
campesterol	19.70	-
stigmasterol	20.59	-
β -sitosterol	23.37	-
Compound II	19.13	12.68
	20.30	56.33
	23.25	30.99

The mass spectrum (Fig. 13) displayed the molecular ion peak at $m/e = 414$, 412 and 400 which corresponded to the molecular ion peaks of β -sitosterol ($\text{C}_{29}\text{H}_{50}\text{O}$), stigmasterol ($\text{C}_{29}\text{H}_{48}\text{O}$) and campesterol ($\text{C}_{28}\text{H}_{48}\text{O}$), respectively. The fragmentation of Compound II was observed at $m/e = 396$, 382, 329, 300, 273, 271, 255, 213, 159, 105, 83 and 55, the fragmentation pattern which was similar to β -sitosterol, stigmasterol and campesterol as shown in Scheme 2 (23).

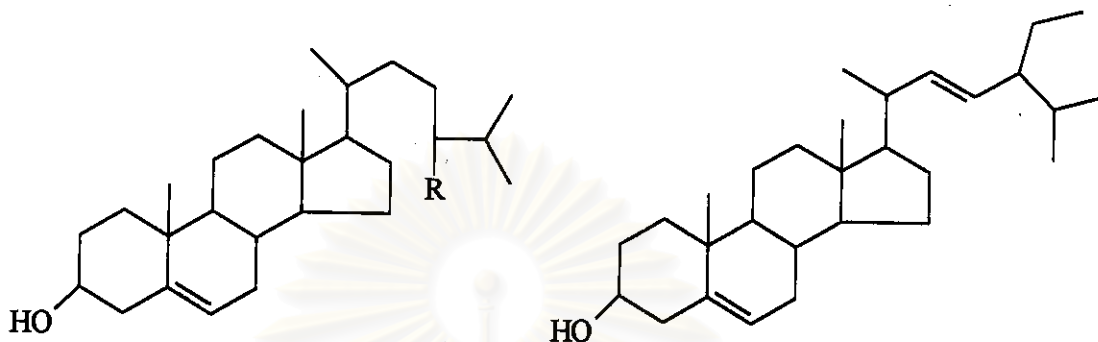


Scheme 2 The Fragmentation Pattern of Compound II.



Scheme 2 The Fragmentation Pattern of Compound II (continued).

From the fragmentation pattern, it was possible to confirm that Compound II was a mixture of β -sitosterol, stigmasterol and campesterol. Structures of these mixture were shown below :



R = CH₃ : Campesterol

R = C₂H₅ : β -Sitosterol

Stigmasterol

Structural Elucidation of Compound III

Compound III was obtained from silica gel column chromatography of hexane crude extract. It was further purified by recrystallization from hexane/chloroform for several times to give 29.9 mg as a white amorphous solid (equivalent to 0.16 % wt. by wt. of hexane crude extract). Its melting point was 73-75 °C and its R_f value was 0.77 (silica gel, 10 % MeOH/CHCl₃). This compound was soluble in dichloromethane, chloroform, ethyl acetate and methanol.

The IR spectrum is shown in Fig. 14 and the absorption bands were assigned in Table 13.

Table 13 The IR Absorption Band Assignments of Compound III.

Wavenumber (cm^{-1})	Intensity	Vibration
2800-3600	s	O-H stretching vibration of carboxylic acids
2919, 2851	s, s	C-H stretching vibration of $-\text{CH}_2-$, $-\text{CH}_3$
1715	m	C=O stretching vibration of carbonyl group of carboxylic acids
1471	m	C-H bending vibration of $-\text{CH}_2-$ and asymmetric bending vibration of $-\text{CH}_3$
1262	w	C-O stretching vibration
721	w	C-C bending vibration of $(-\text{CH}_2-)_n$; $n \geq 4$

The IR spectrum revealed the presence of carboxyl group as evidenced by the absorption band at 1715 cm^{-1} of C=O stretching vibration peak, at 1262 cm^{-1} of C-O stretching vibration peak and at $2800-3600 \text{ cm}^{-1}$ of O-H stretching vibration peak. The C-H stretching vibration peaks of an aliphatic compounds were observed at 2919 and 2851 cm^{-1} . The medium intensity absorption peak at 1471 cm^{-1} corresponded to C-H bending vibration mode of $-\text{CH}_2-$, $-\text{CH}_3$ and the absorption peak at 721 cm^{-1} revealed mode of saturated long chain of $(-\text{CH}_2-)_n$; $n \geq 4$.

From $^1\text{H-NMR}$ spectrum (Fig. 15) the signal of methyl protons ($-\text{CH}_3$) appeared as triplet at $\delta 0.86 \text{ ppm}$ and signal of methylene protons $[(-\text{CH}_2-)_n]$ appeared at $\delta 1.24 \text{ ppm}$. The signal at $\delta 1.61 \text{ ppm}$ were β - CH_2- protons and at $\delta 2.33 \text{ ppm}$ were α - CH_2- protons.

The $^{13}\text{C-NMR}$ spectrum (Fig. 16) showed a signal of carbon in the methyl group, $-\text{CH}_3$, at $\delta 14.10 \text{ ppm}$ and the signals of methylene group, $(-\text{CH}_2-)_n$, at $\delta 22.68-29.69 \text{ ppm}$. At $\delta 31.92$ and 33.94 ppm , signals of β - CH_2- and α - CH_2- were observed, respectively. A signal of the carbonyl functional group of carboxylic acids was evidenced at $\delta 179.49 \text{ ppm}$.

IR, $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectral data suggested that Compound III should be a long chain carboxylic acid or a mixture of acids. GC technique was used to confirm this suggestion. The gas chromatogram appeared as eight peaks at different retention times as shown in Table 14 and Fig. 17. By comparison to standard long chain carboxylic acids using calibration curve of log retention time with number of carbon (Fig. 18). Compound III was shown to be a mixture of eight long chain carboxylic acids ($\text{C}_{18, 22-34}$).

Table 14 The Retention Time and Log Retention Time of Compound III

Retention time (min)	Log retention time	Number of carbon
2.19	0.34	18
3.53	0.55	22
4.57	0.66	24
5.93	0.77	26
7.75	0.89	28
10.09	1.00	30
13.22	1.12	32
17.33	1.24	34

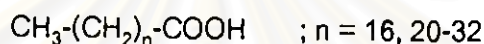
Table 15 Name of Long Chain Aliphatic Carboxylic Acids in Compound III.

Molecular Formula	Molecular Weight	Name of Substance	% Composition
$\text{C}_{18}\text{H}_{36}\text{O}_2$	284	octadecanoic acid	1.39
$\text{C}_{22}\text{H}_{44}\text{O}_2$	340	docosanoic acid	29.48
$\text{C}_{24}\text{H}_{48}\text{O}_2$	368	tetracosanoic acid	31.12
$\text{C}_{26}\text{H}_{52}\text{O}_2$	396	hexacosanoic acid	21.42
$\text{C}_{28}\text{H}_{56}\text{O}_2$	424	octacosanoic acid	8.79
$\text{C}_{30}\text{H}_{60}\text{O}_2$	452	triacontanoic acid	3.87

Table 15 Name of Long Chain Aliphatic Carboxylic Acids in Compound III (continued).

Molecular Formula	Molecular Weight	Name of Substance	% Composition
$C_{32}H_{64}O_2$	480	dotriacontanoic acid	2.29
$C_{34}H_{68}O_2$	508	tetratriacontanoic acid	1.63

The major components of Compound III were $C_{24}H_{48}O_2$ and $C_{22}H_{44}O_2$, 31.12 % and 29.48 %, respectively. The structure of Compound III was thus presented below :



Structural Elucidation of Compound IV

Compound IV was isolated from dichloromethane crude extract and ethyl acetate crude extract. This compound was colourless needles (18.3 mg) with a melting point of 206-208 °C and R_f value of 0.17 (silica gel, 1 % MeOH/ $CHCl_3$). It was soluble in dichloromethane, chloroform, ethyl acetate and methanol and insoluble in hexane.

The IR spectrum of Compound IV (Fig. 19) were assigned in Table 16. The IR spectrum indicated hydroxy group (O-H stretching vibration peak) at 3000-3600 cm^{-1} , C=O stretching vibration peaks at 1714 cm^{-1} , the C-O asymmetric stretching vibration peak at 1263 cm^{-1} and at 1102 and 1021 cm^{-1} which corresponded to C-O symmetric vibration peaks. The C-H stretching vibration of aromatic compounds and aliphatic compounds were observed at 2959, 2927 and 2873 cm^{-1} , respectively. The peaks at 1611, 1568, 1509 and 1458 cm^{-1} indicated the C=C stretching vibration of aromatic compounds and the =C-H out of plane bending vibration was observed at 862 and 804 cm^{-1} .

Table 16 The IR Absorption Band Assignments of Compound IV.

Wavenumber (cm ⁻¹)	Intensity	Vibration
3000-3600	s	O-H stretching vibration
2959	s	C-H stretching vibration of aromatic compounds
2927, 2873	s, s	C-H stretching vibration of -CH ₃
1714	s	C=O stretching vibration
1611, 1568, 1509, 1458	m, m, m, m	C=C stretching vibration of aromatic compounds
1263	m	C-O asymmetric stretching vibration
1102, 1021	m, m	C-O symmetric stretching vibration
862, 804	m, m	=C-H out of plane bending vibration

From ¹H-NMR spectrum (Fig. 20) the signal at δ 3.93 ppm revealed proton of O-CH₃ group as a singlet. The signals at δ 6.82 and 6.90 ppm were protons of aromatic ring (=CH-) and signals at δ 6.19 and 7.54 ppm were olefinic protons (-CH=).

From ¹³C-NMR spectrum (Fig. 21), DEPT 90 (Fig. 22) and DEPT135 (Fig. 23) the signal at δ 56.48 ppm was sp³ carbon linked to oxygen atom, and was probably a methoxy group (O-CH₃). The signals at δ 103.25, 107.84, 113.43 and 142.88 ppm were sp² carbons of =CH- group, the signals at δ 111.52, 144.12, 149.90 and 150.46 ppm were quaternary sp² carbons and the signal at δ 161.00 ppm was sp² carbon of carbonyl functional group of coumarin compounds.

From data of ¹H-NMR and ¹³C-NMR spectra, Compound IV was similar to scopoletin (7-hydroxy-6-methoxycoumarin). The signals of ¹H-NMR and ¹³C-NMR spectra of these compounds were compared as shown in Table 17.

Table 17 Comparison of $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ Spectra of Compound IV to Scopoletin (24) and Scopolin (25).

Position	Compound IV		Scopoletin		Scopolin	
	δ_{H} (ppm) ^a	δ_{C} (ppm) ^a	δ_{H} (ppm) ^b	δ_{C} (ppm) ^b	δ_{H} (ppm) ^c	δ_{C} (ppm) ^c
2	-	161.00 (s)	-	-	-	160.9 (s)
3	6.19(d)	113.43 (d)	6.10 (d)	-	6.12 (d)	114.1 (d)
4	7.54(d)	142.88 (d)	7.60 (d)	-	7.19 (d)	143.5 (d)
5	6.90 (s)	107.84 (d)	6.90 (s)	-	6.83 (s)	110.3 (d)
6	-	144.12 (s)	-	-	-	147.2 (s)
7	-	149.90 (s)	-	-	-	151.3 (s)
8	6.82 (s)	103.25 (d)	6.70 (s)	-	7.03 (s)	104.3 (d)
9	-	150.46 (s)	-	-	-	150.1 (s)
10	-	111.52 (s)	-	-	-	113.0 (s)
OCH ₃	3.93 (s)	56.48 (q)	3.80 (s)	-	3.52(s)	56.5 (q)

^a Measured in CDCl₃.

^b Measured in DMSO-d₆ + CDCl₃.

^c Measured in pyridine-d₅.

By comparison chemical shift of protons and carbons of Compound IV to scopoletin (24) and scopolin (scopoletin glucoside) (25) (Fig. 4), the spectra show close similarity as in Table 17. Moreover, melting point of Compound IV (206-208 °C) was similar to melting point of scopoletin (204 °C) (26).

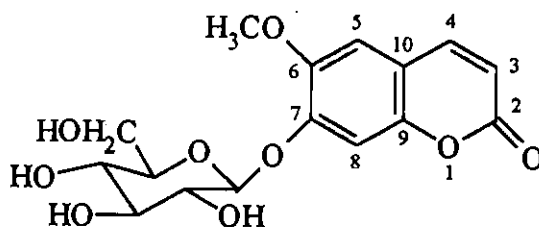
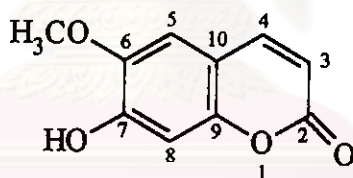


Figure 4 The Structure of Scopolin.

The mass spectrum (Fig. 24) displayed the molecular ion peak at m/e 192 and other fragmentation at m/e 177($M^+ - CH_3$), 164($M^+ - CO$), 149(177-CO), 135($M^+ - C_2H_5C=O$) and 121($M^+ - C_3H_3O_2$). This fragmentation pattern was similar to fragmentation pattern of scopoletin (Fig. 25).

From the spectroscopic data, it can be concluded that Compound IV was scopoletin (7-hydroxy-6-methoxycoumarin). The structure of scopoletin which was shown below :



Structural Elucidation of Compound V

Compound V was a white amorphous solid from dichloromethane crude extract and ethyl acetate crude extract and weighed 203.8 mg (154.1 mg = 0.82 % wt. by wt. of dichloromethane crude extract and 49.7 mg = 0.24 % wt. by wt. of ethyl acetate crude extract). This solid was purified by recrystallization from chloroform/methanol. Its melting point was 271-273 °C and the R_f value was 0.16 (silica gel, 10 % MeOH/ $CHCl_3$). Compound V was slightly soluble in chloroform, ethyl acetate and methanol and was insoluble in hexane.

The IR spectrum of this compound was presented in Fig. 26 and the absorption band assignments were shown in Table 18. Its IR spectrum strongly vibration indicated O-H stretching vibration at $3100-3600\text{ cm}^{-1}$ and C=C stretching vibration peak at 1651 cm^{-1} . The C-O stretching vibration of glycosidic linkage was observed at $1025-1074\text{ cm}^{-1}$ and an anomeric axial C-H deformation of β -sugar was observed at 804 cm^{-1} .

The results of IR spectrum indicated that Compound V might be a glycoside.

Table 18 The IR Spectrum Absorption Band Assignments of Compound V.

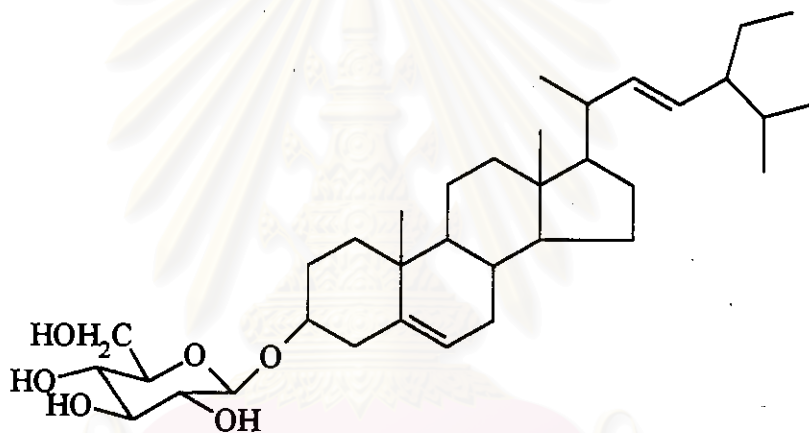
Wavenumber (cm^{-1})	Intensity	Vibration
3100-3600	s	O-H stretching vibration
2954	s	C-H stretching vibration of =CH-
2936, 2868	s, s	C-H stretching vibration of $-\text{CH}_2-$, $-\text{CH}_3$
1651	w	C=C stretching vibration
1462	m	C-H bending vibration of $-\text{CH}_2-$ and asymmetric bending vibration of $-\text{CH}_3$
1371	m	C-H symmetric bending vibration of $-\text{CH}_3$
1261	m	C-O stretching vibration
1025-1074	s	C-O stretching vibration of glycosidic linkage
804	m	anomeric axial C-H deformation of β -sugar

The $^1\text{H-NMR}$ spectrum (Fig. 27) exhibited signals at $\delta 0.64-2.51\text{ ppm}$ of methyl and methylene protons of steroid. The doublet signals at $\delta 4.20\text{ ppm}$ revealed anomeric protons of β -D-glucose. The signals at $\delta 2.90-3.74\text{ ppm}$ was protons of sugar and the signal at $\delta 4.04\text{ ppm}$ was proton of CH-O-sugar . The alkene protons of steroid appeared at $\delta 4.87, 5.07\text{ ppm}$ ($\text{CH}=\text{CH}$) and 5.32 ppm ($\text{CH}=\text{C}$).

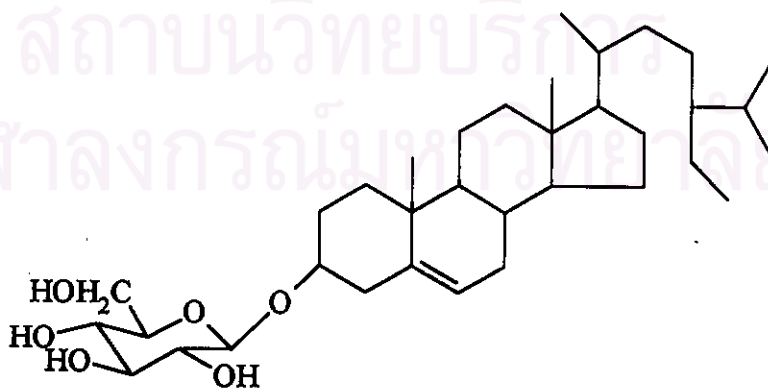
The ^{13}C -NMR spectrum (Fig. 28) showed the signals of glucose at δ 61.04, 70.03, 73.42, 76.71, 76.91 and 100.78 ppm and other signals corresponded to signals of steroid which was similar to Compound II (Fig. 11).

The mass spectrum (Fig. 29) did not show molecular ion peaks. The dominant fragmentation were found at m/e 414, 412, 400, 396, 394, 382, 351, 300, 225, 213, 159, 145, 95, 83, 69 and 55. This fragmentation pattern was similar to the fragmentation of compound II (scheme 2).

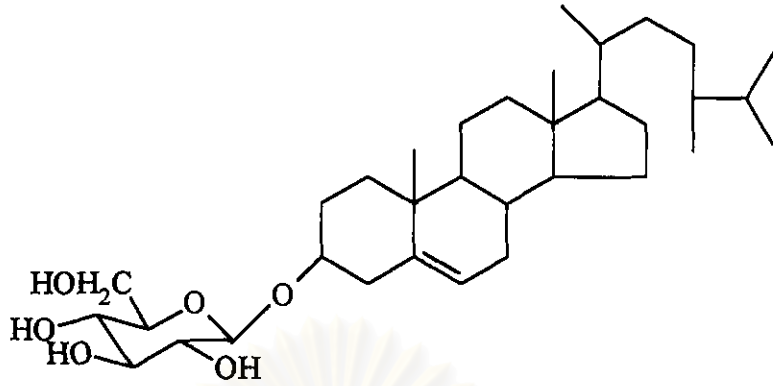
From all the data, Compound V was a mixture of stigmasteryl-3-O- β -D-glucopyranoside, β -sitosteryl-3-O- β -D-glucopyranoside and campesteryl-3-O- β -D-glucopyranoside. The structures of this mixture are shown below :



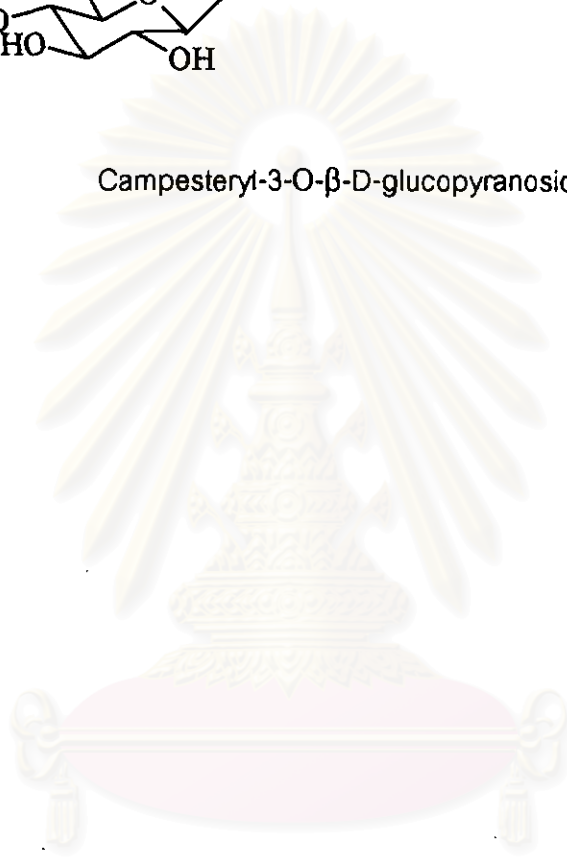
Stigmasteryl-3-O- β -D-glucopyranoside



β -Sitosteryl-3-O- β -D-glucopyranoside



Campesteryl-3-O-β-D-glucopyranoside



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