CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The stem bark of *Michelia longifolia* Blume (*M. alba* DC.) was collected from Uttradit Province, Thailand, in July 1986. The plant material was identified by comparison with a specimen in medicinal plant garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Analytical

Technique	:	one way, ascending
Absorbent	:	Silica gel G (E. Merck); 30 g./60 ml of distilled water
Plate Size	:	5 cm x 20 cm, 10 cm x 20 cm and 20 cm x 20 cm
Layer Thickness	:	0.25 mm
Activation	:	air dried for 15 minutes and then heat at 105 °C for 1 hour.
Solvent Systems.	:	a) Chloroform : Acetone (9:1) b) Benzene : Ethyl Acetate (9:1) c) Chloroform : Ethyl Acetate (7:3) d) Methylene Dichloride e) Chloroform : Acetone (7:3)

Distance : 15 cm

Laboratory Temperature : 24-30° C

Detection on Chromatographic Plate :

1) Ultraviolet light at 365 nm.

The alkaloids became visible as yellow fluorescent spots under UV light at 365 nm

2) Developing reagents

a) 2 % methanolic solution of resorcin mixed with 2 % H_2SO_4 solution (1:1)

The spots of sesquiterpene lactones gave specific colors with this reagent after heating at 110°C for 2-4 min.

b) Modified Dragendroff's spray reagent

Solution A : Bismuth subnitrate (850 mg), distilled water (40 ml) and glacial acetic acid (10 ml)

Solution B : Potassium iodide (8 g) and distilled water (20 ml)

Solution A and B, 5 ml each, were mixed, 20 ml of glacial acetic acid and 70 ml of distilled water were added and used as spray reagent. The alkaloids gave orange or yellowish orange spots with the reagent.

2.2 Column Chromatography (CC)

Column Size	:	2.5 cm x 50 cm, 4 cm x 50 cm
Adsorbent	:	silica gel 0.040-0.063 mm (E. Merck)
Packing of Column	:	dry packing
Sample loading	:	the portion of crude extract was dissolved in small amount of organic solvent, mixed with small quantity of adsorbent, dried, triturated and added onto the top of a column.
Solvents	:	a) Chloroform : Acetone (9:1) b) Benzene : Ethyl Acetate (9:1) c) Chloroform : Ethyl Acetate (7:3) d) Methylene Dichloride e) Chloroform : Acetone (7:3)

2.3 Physical Constants

2.3.1 Melting points

Melting points were determined by Gallenkemp melting point apparatus.

2.3.2 Specific rotations

Specific rotations were performed in a Bendix-NPL automatic polarimeter.

2.4 Spectroscopy

2.4.1 Nuclear Magnetic Resonance Spectra

^{1}H-nmr spectra were recorded on a Bruker WH

400 spectrometer with TMS (δ = 0) as internal standard with solvents as indicated.

2.4.2 Mass spectra

Mass spectra were obtained on a Varian MAT CH 7 or VG Micromass 7070 F spectrometer.

2.4.3 Infrared spectra

IR spectra were obtained on a Perkin-Elmer Model 1330 or 180 spectrophotometer.

2.4.4 Ultraviolet spectra

UV spectra were obtained on a Varian DMS 90 spectrophotometer.

2.5 Authentic samples

Parthenolide and liriodenine obtained from *Paramichelia baillonii* (Pierre) Hu were kindly supplied by Mr. Arthorn Rivepiboon.

β-Sitosterol obtained from *Typha elephantina* Roxb. was kindly supplied by Ms. Arunporn Aukkanibutra.

3. Isolation of Chemical Substances from *Michelia longifolia* Bl. stem bark.

3.1 Extraction

The fresh stem bark of *Michelia longifolia* Bl. (2.5 kg) was blended with 95 % ethanol (10 L), macerated twice over a period of 3 days, and then filtered. The filtrate was evaporated under reduced

pressure to yield a syrupy mass (81.2 g). The syrupy mass was suspended in distilled water (150 ml), extracted with chloroform (5 x 300 ml), dried (Na_2SO_4 anhydrous) and evaporated under reduced pressure to dryness to yield the crude extract (10 g).

3.2 Isolation of Chemical Substances

The crude extract (10 g) was divided into four equal portions. Each portion was chromatographed over a silica gel column (4 cm X 50 cm) using chloroform : acetone (9:1) as the eluent. Fractions of 20 ml. were collected and examined by thin layer chromatography (TLC). Those fractions of similar pattern were combined and yielded 2 major fractions as follows :-

a) Fractions 4-22 afforded a crude mixture (0.32 g) which was further purified using benzene : ethyl acetate (9:1) as the eluent. Fractions of 20 ml. were collected and combined as follows :-

i) Fractions 3-7 which was further chromatographed over a silica gel column using methylene dichloride as the eluent to yield a white amorphous powder of ML-4, 58 mg.

ii) Fractions 8-11 which was further purified using chloroform : ethyl acetate (7:3) as the eluent to yield a white amorphous powder of ML-2, 29 mg.

iii) Fractions 12-24 which was evaporated under reduced pressure to dryness and crystallized in petroleum ether to yield clear colorless cubic crystals of ML-1, 106 mg.

b) Fractions 23-65 from the first column was rechromatographed over a silica gel column using chloroform : acetone (7:3) as the eluent to afford yellow rosette crystals of ML-3, 14 mg.

4. Characterization of ML-1, ML-2, ML-3 and ML-4

ML-1, ML-2, ML-3 and ML-4 were characterized by studies on color reactions, melting points, ultraviolet, infrared, nuclear magnetic resonance and mass spectra. The hRf values given are obtained from the following systems :-

a) silica gel G/chloroform : acetone (9:1)

b) silica gel G/benzene : ethyl acetate (9:1)

c) silica gel G/chloroform : ethyl acetate (7:3)

4.1 Characterization of ML-1 as Parthenolide

ML-1 was obtained as clear colorless cubic crystals. It was soluble in ether, acetone, chloroform and ethyl alcohol.

hRf values

a) 52.7 (Fig. 3.1) b) 18.0 (Fig. 3.2) c) 45.3 (Fig. 3.3)

Color Reaction

ML-1 gave cherry red color with 2 % methanolic solution of resorcin mixed with 2 % sulfuric acid solution (1:1) on TLC (silica gel G) plate.

Melting Point

112-115°C (uncorrected)

Optical Rotation

$(\alpha)_D^{20}$ = - 76 ° ($CHCl_3$)

Molecular weight

248 (EIMS)

Infrared Absorption Spectrum (CCl_4)

$\nu_{max}(cm^{-1})$

3020, 2920, 1770, 1650, 1281, 1260, 1130 and 940 cm^{-1}.

(Figure 3.4, p. 133)

NMR Spectrum

The NMR spectrum was performed in deuterochloroform at 400 MHz in δ value (ppm) from tetramethylsilane (T.M.S.).

(Figure 3.5, p. 134)

Proton	Multiplicity	Chemical Shift (δ) ppm	Coupling Constant (J) Hz
1 (1H)	dd, br	5.21	4.0, 12.2
2α (1H)	dd	2.09-2.38	5.1, 13.1
2β (1H)	ddd	2.46	13.8, 12.2, 12.5
3α (1H)	m	1.25	
3β (1H)	m	2.09-2.17	
5 (1H)	d	2.79	8.9
6 (1H)	dd	3.86	8.9, 8.3
7 (1H)	m	2.78	

Proton	Multiplicity	Chemical Shift	Coupling Constant
8α (1H)	m	2.09-1.72	
8β (1H)	m	1.73	
9α (1H)	m	2.09-2.24	
9β (1H)	m	2.38	
13a (1H)	d	6.38	3.6
13b (1H)	d	5.62	3.1
14-CH_3 (3H)	s	1.72	
15-CH_3 (3H)	s	1.31	

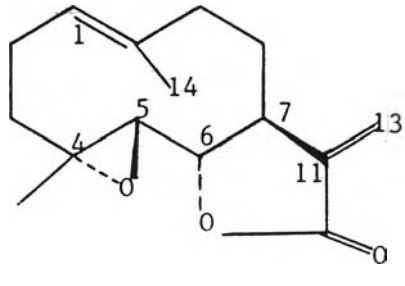
Mass Spectrum (EIMS)

m/z (% relative intensity)

248 (M^+, 2), 230 (9), 191 (25), 190 (61), 119 (100)

(Figure 3.6, p. 135)

These data were identical with those obtained for a sample of parthenolide, which had been isolated from *Paramichelia baillonii* (94), and also were in accord with those published previously for this lactone (94, 6). Thus ML-1 is concluded to have the structure shown below :-



ML-1

PARTHENOLIDE

4.2 Characterization of ML-2 as β-Sitosterol

ML-2 was obtained as a white amorphous powder. It was soluble in petroleum ether, ether, acetone and chloroform.

hRf Value

a) 28.0 (Fig. 3.1) b) 29.3 (Fig. 3.2) c) 36.0 (Fig. 3.3)

Color Reaction

ML-2 gave orange color with 2 % methanolic solution of resorcin mixed with 2 % sulfuric acid solution (1:1) on TLC (silica gel G) plate.

Melting Point

134-135 °C

Molecular Weight

414 (EIMS)

Infrared Absorption Spectrum (KBr disc)

ν_{max} (cm^{-1})

3520 (broad), 2950, 2850, 1650, 1450, 1390, 1380, 1060, 1020 and 800 cm^{-1}

(Figure 3.7, p, 136)

NMR Spectrum

The NMR spectrum was performed in deuterochloroform at 400 MHz in δ value (ppm) from tetramethylsilane (T.M.S.)

(Figure 3.8, p. 137)

Proton	Multiplicity	Chemical Shift (δ) ppm
18-CH_3 (3H)	s	0.68
29-CH_3(3H)	t	0.78
26,27-CH_3 (6H)	d	0.82
21-CH_3(3H)	d	0.92
19-CH_3 (3H)	s	1.01
3-H (1H)	m	3.5
6-H (1H)	t	5.3

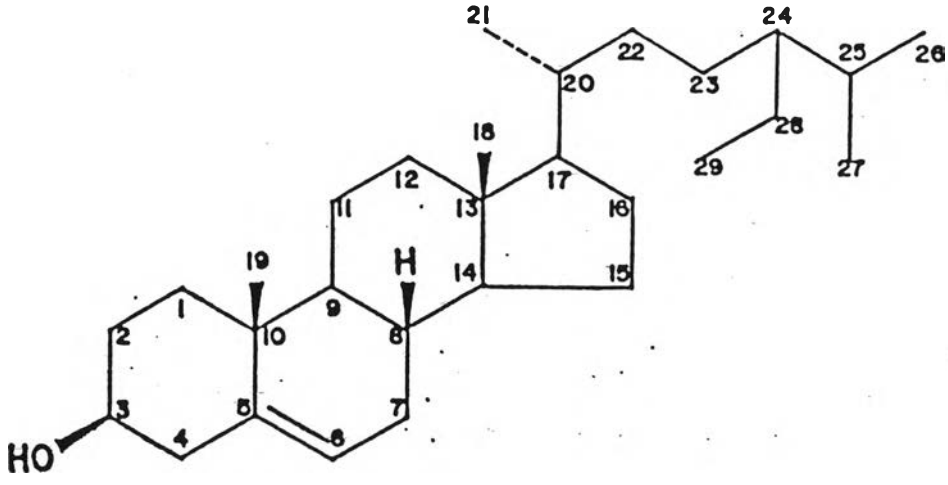
Mass Spectrum (EIMS)

m/z (% relative intensity)

414 (M^+, 85), 399 (21), 396 (100), 381 (36), 329 (29), 273 (25), 255 (61), 231 (19), 213 (41), 173 (21), 163 (37), 161 (47), 159 (58), 147 (66), 145 (85), 135 (41), 133 (55), 131 (43), 121 (53), 119 (51), 109 (48), 107 (75), 105 (79), 95 (75), 93 (64), 91 (65), 83 (50), 81(100), 43(92).

(Figure 3.9, p. 138)

These data were identical with those obatined for a sample of β-sitosterol, which had been isolated from *Typha elephantina* (97), and also in accord with those published previously for this sterol (96 , 97). Therefore it is concluded that ML-2 is β-sitosterol, the structure of which is shown on page 103.



ML-2

β-SITOSTEROL

4.3 Characterization of ML-3 as Liriodenine

ML-3 was obtained as yellow rosette crystals. It was slightly soluble in chloroform, soluble in ethyl alcohol and insoluble in petroleum ether.

hRf Value

a) 18.7 (Fig. 3.1) b) 3.3 (Fig. 3.2) c) 13.3 (Fig. 3.3)

Color Reaction

ML-3 gave orange color with Dragendroff's reagent

Melting Point

278-282 °C

Molecular Weight

275 (EIMS)

Infrared Absorption Spectrum (CH_2Cl_2)

ν_{max} (cm^{-1})

3040, 2920, 1655, 1590, 1480, 1462, 1438, 1410, 1300, 1220, 1050, 1010, 965, 890, 865 cm^{-1}

(Figure 3.10, p.139)

Ultraviolet Absorption Spectra

$\lambda_{max}^{(EtOH)}$ 250, 270, 310, 400 (sh), 416 nm (Figure 3.14, p. 143)

$\lambda_{max}^{(0.1NHCl\ in\ EtOH)}$ 260, 282, 320, 396, 452 nm (Figure 3.15, p. 144)

NMR Spectrum

The NMR spectra were performed in deuterochloroform and 10 % DMSO-d_6 in deuterochloroform at 400 MHz in δ value (ppm) from tetramethylsilane (T.M.S.).

$CDCl_3$ (Figure 3.11, p. 140)

Proton	Multiplicity	Chemical Shift (δ)	Coupling Constant (J) Hz
3	s	7.17	
4	br, s	7.74	
5	br, s	8.80	
8	d	8.58	8.1
9	t	7.57	8.0
10	t	7.73	8.0
11	d	8.61	8.1
-O-CH_2-O-	s	6.37	

10 % DMSO-d_6 in $CDCl_3$ (Figure 3.12, p. 141)

Proton	Multiplicity	Chemical Shift (δ)	Coupling Constant (*J*) Hz
3	s	7.21	
4	br,s	7.83	
5	br,s	8.90	
8	d	8.57	8.0
9	t	7.58	8.0
10	t	7.77	8.0
11	d	8.72	8.0
$-O-CH_2-O-$	s	6.41	

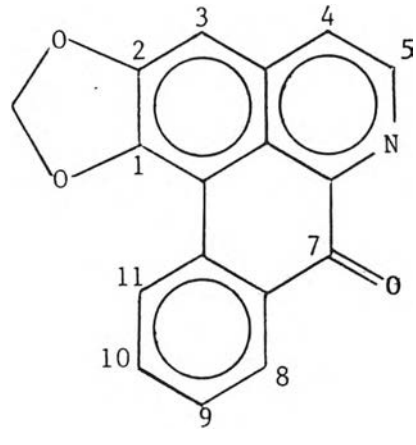
Mass Spectrum (EIMS)

m/z (% relative intensity)

275 (M^+, 80), 247 (14), 246 (10)

(Figure 3.13, p. 142)

According to all spectral data and direct comparison with an authentic sample(94), it is clearly proved that ML-3 is liriodenine (6), the structure of which is shown on page 106.



ML-3

LIRIODENINE

4.4 Characterization of ML-4 as Costunolide

ML-4 was obtained as a white amorphous powder. It was soluble in ether, acetone, chloroform and ethyl alcohol.

hRf Value

a) 62.7 (Fig. 3.1) b) 50.0 (Fig. 3.2) c) 54.0 (Fig. 3.3)

Color Reaction

ML-4 gave violet color with 2 % methanolic solution of resorcin mixed with 2 % sulfuric acid (1:1) on TLC (silica gel G) plate.

Melting Point

105-106° C

Optical Rotation

$(\alpha)_D^{20}$ = + 125° ($CHCl_3$)

Infrared Absorption Spectrum (CCl_4)

ν_{max} (cm^{-1})

2932, 1772, 1289, 1244, 1137, 971 cm^{-1}

(Figure 3.16, p. 145)

NMR Spectrum

The NMR spectrum was performed in deuterochloroform at 400 MHz in δ value (ppm) from tetramethylsilane (T.M.S.).

(Figure 3.17, p. 146)

Proton	Multiplicity	Chemical Shift (δ)	Coupling Constant (J) Hz
1 (1H)	br, d	4.83	
2α (1H)	m	1.69-2.98	
2β (1H)			
3α (1H)			
3β (1H)			
5 (1H)	d	4.79	
6 (1H)	t	4.54	10
7 (1H)	m	2.58	
8α (1H)	m	1.69-2.98	
8β (1H)			
9α (1H)			
9β(1H)			
13a (1H)	d	6.24	3
13b (1H)	d	5.52	3
14-CH_3 (3H)	d	1.69*	1
15-CH_3 (3H)	d	1.43*	1

* Assignments may be interchanged.

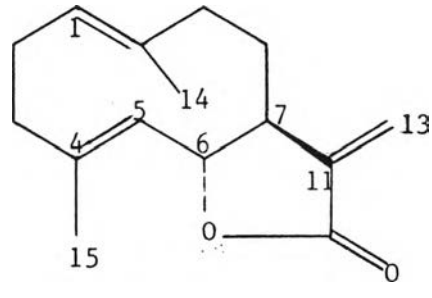
Mass Spectrum (EIMS)

m/z (% relative intensity)

232 (M^+, 20), 217 (17), 150 (15), 123 (48), 121 (36), 119 (19), 109 (62), 107 (28), 105 (32), 95 (23), 93 (31), 91 (23), 81 (100), 80 (27), 79 (30), 53 (31).

(Figure 3.18, p. 147)

All the above data are in accord with those published previously for costunolide (6). Thus ML-4 is concluded to be costunolide unambigously. The structure is shown below :-



ML-4

COSTUNOLIDE