CHAPTER III

EXPERIMENTAL

Source of Plant Material

The stem bark of *Croton oblongifolius* used in this study was collected from amphur Hun Ka, Chainat province, Thailand on May 17, 1998. The plant was identified by comparison with the herbarium specimen no.BKF 084729 deposited at the Royal Forest Department of Thailand.

General Techniques

1. Chromatography

1.1. Analytical Thin-layer Chromatography (TLC)

Technique	: One dimension, ascending
Adsorbent	: Silica gel 60 F_{254} precoated plate (E. Merck)
Layer thickness	: 0.25 mm.
Developing distance	: 5 cm.
Temperature	: Laboratory temperature (30-35°C)
Detection	: 1.Ultraviolet light at wavelengths of 254 and 365 nm. 2.Iodine vapour 3.Anisaldehyde-H_2SO_4, and heated at 100-105°C for a few minutes

1.2. Column Chromatography

1.2.1. Conventional Column Chromatography

Adsorbent : 1. Silica gel 60 (No.7734) (E.Merck)
particle size 0.063-0.200 nm. (70- 230 mesh ASTM)
2. Silica gel 60 (No.9385) (E.Merck)
particle size 0.040-0.063 nm. (230- 400 mesh ASTM)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount of eluent, then apply gently on top of the column.

Examination of eluate: Fractions were examined by TLC observing under UV light at wavelengths of 254 and 365 nm. The TLC plate was then detected by exposing to iodine vapor, and by exposing to Anisaldehyde-H_2SO_4 reagent respectively. Fractions of similar chromatographic pattern were combined.

1.2.2. Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

Packing method : Gel filter was suspended in the eluent and left standing to swell for 24 hours before using, then poured into the column and allowed to settle properly.

Sample loading : The sample was dissolved in a small volume of eluent and applied on top of the column.

Examination of eluate: Fractions were examined in the same manner as described in section 1.2.1.

1.2.3. Flash Column Chromatography

Adsorbent : Polystyrene (MCI Gel)
High Porous Polymer CH20P (75-150μ)
Mitsubishi Chemical Corporation

Packing method: The adsorbent was wet-packed after being suspended in methanol. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump, then allowed to settle.

Sample loading : The sample was dissolved in a small volume of eluent and applied on top of the column.

Examination of eluate: Fractions were examined in the same manner as described in section 1.2.1.

1.3. Gas chromatography

GC analysis were performed on a Shimadzu Gas Chromatograph Model GC-7AG (The Scientific and Technology Research Equipment Center, Chulalongkorn University).

2. Spectroscopy

2.1. Ultraviolet (UV) Absorption Spectra

UV spectra were obtained from a Shimadzu UV-160A UV/vis spectrophotometer (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.2. Infrared (IR) Absorption Spectra

IR (KBr disc) spectra were carried out on a Perkin-Elmer FT-IR spectrum 2000 Spectrophotometer (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3. Mass Spectra (MS)

EIMS of isolated compounds were performed with a Micromass (VG Platform II, Fisons Instrument) Spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4. Nuclear Magnetic Resonance (NMR) Spectra

The NMR spectra were obtained on a JEOL JMN-A500 (Alpha series) 500 MHz NMR Spectrometer (The Scientific and Technology Research Equipment Center, Chulalongkorn University) and an Avance DPX-300 FT-NMR Spectrometer, Bruker Spectro-Spin (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

NMR solvents used in this study were deuterated chloroform (chloroform-*d*) with tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

3. Physical Properties

3.1. Melting point

Melting points were obtained on a Gallenkamp Melting Point Apparatus (Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.2. Optical rotation

Optical rotations were measured on a Perkin-Elmer Polarimeter model 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

4. Solvents

Throughout this work, all organic solvents, excluding the deuterated solvent for NMR spectra, were commercial grade and were redistilled prior to use.

Extraction

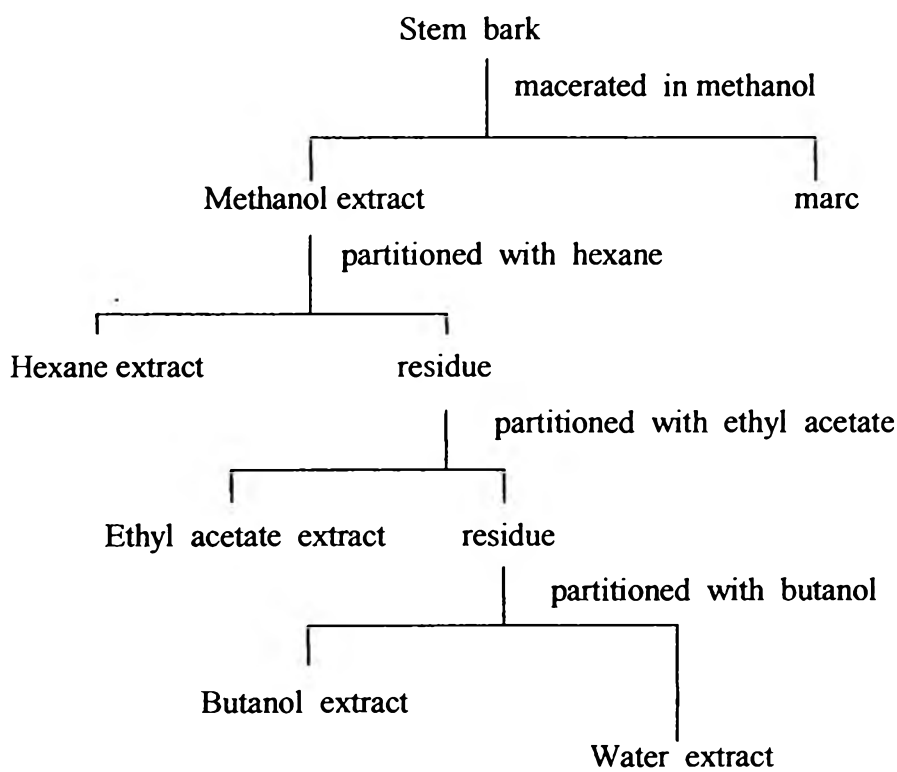
The dried powdered stem bark of *Croton oblongifolius* (8 kg) was repeatedly extracted by maceration with methanol (15 lit,.2 days, 3 times). Methanol extract was filtered. The filtrate was evaporated under reduced pressure to give a dark-red gummy residue which was then repeatedly partitioned with hexane, ethyl acetate and butanol, respectively.

The hexane layer was evaporated to yield yellow-green oil (430 g: 5.38% w/w based on dried weight of stem bark).

The ethyl acetate extract was obtained as red-brown residue (124 g: 1.55% w/w based on dried weight of stem bark).

The butanol extract was obtained as brown residue (74 g: 0.93% w/w based on dried weight of stem bark).

The water extract was obtained as red-brown residue (1251 g.: 15.64% w/w based on dried weight of stem bark).



Scheme 1 Extraction of *Croton oblongifolius* stem bark

Isolation

The hexane extract (230 g) was subjected to column chromatography, using silica gel 60 (No.7734), eluted with hexane, hexane-EtOAc, EtOAc and $CHCl_3$. Fractions were collected and combined according to TLC pattern using hexane: EtOAc 3:1 as developing system to give 4 fractions, F001 – F004.

F001 was then fractionated by gel filtration chromatography using Sephadex LH-20, eluted with MeOH. Fractions were collected and combined according to TLC pattern to give 5 fractions, F005 – F009.

Isolation of compound COY4

F006 (175g) yield crystals in hexane at room temperature. The mother liqour was removed and the crystals were recrystallized with hot hexane to give fined white powder, COY4 (4.98 g).

Furthermore, COY4 could be obtained by the following way, F006 (25 g) was dissolved with hexane (200 ml). The solution of the extract was shaken with 5% NaOH solution (200 ml) at room temperature. The aqueous layer was acidified with 10% H_2SO_4 solution, then extracted with hexane, and evaporated. The residue from hexane layer was subjected to column chromatography using silica gel 60 (No.9835). The separation was carried out with gradient elution, using mixtures of hexane and EtOAc. At concentration of 20% EtOAc in hexane it gave colorless crystals, COY4. (1.25 g).

Thus, 6.23 g. of COY4 could be obtained (2.71% w/w based on hexane extract).

Isolation of isolate COY8 and compound COY10

The mother liqour from F006 was rechromatographed by column chromatography using silica gel 60 (No.9385) as adsorbent and 10% EtOAc in hexane as eluent. Fractions were collected and combined according to TLC pattern to yield 6 fractions, F010 – F015.

F011 yielded white needle crystals in hexane at room temperature, COY8 (0.06 g, 0.02% w/w based on hexane extract).

F010 was rechromatographed on a polystyrene flash column using MCI Gel, and $CHCl_3$: MeOH 1:1 as eluent. Fractions were collected and combined according to TLC pattern to give 3 fractions, F016 – F018.

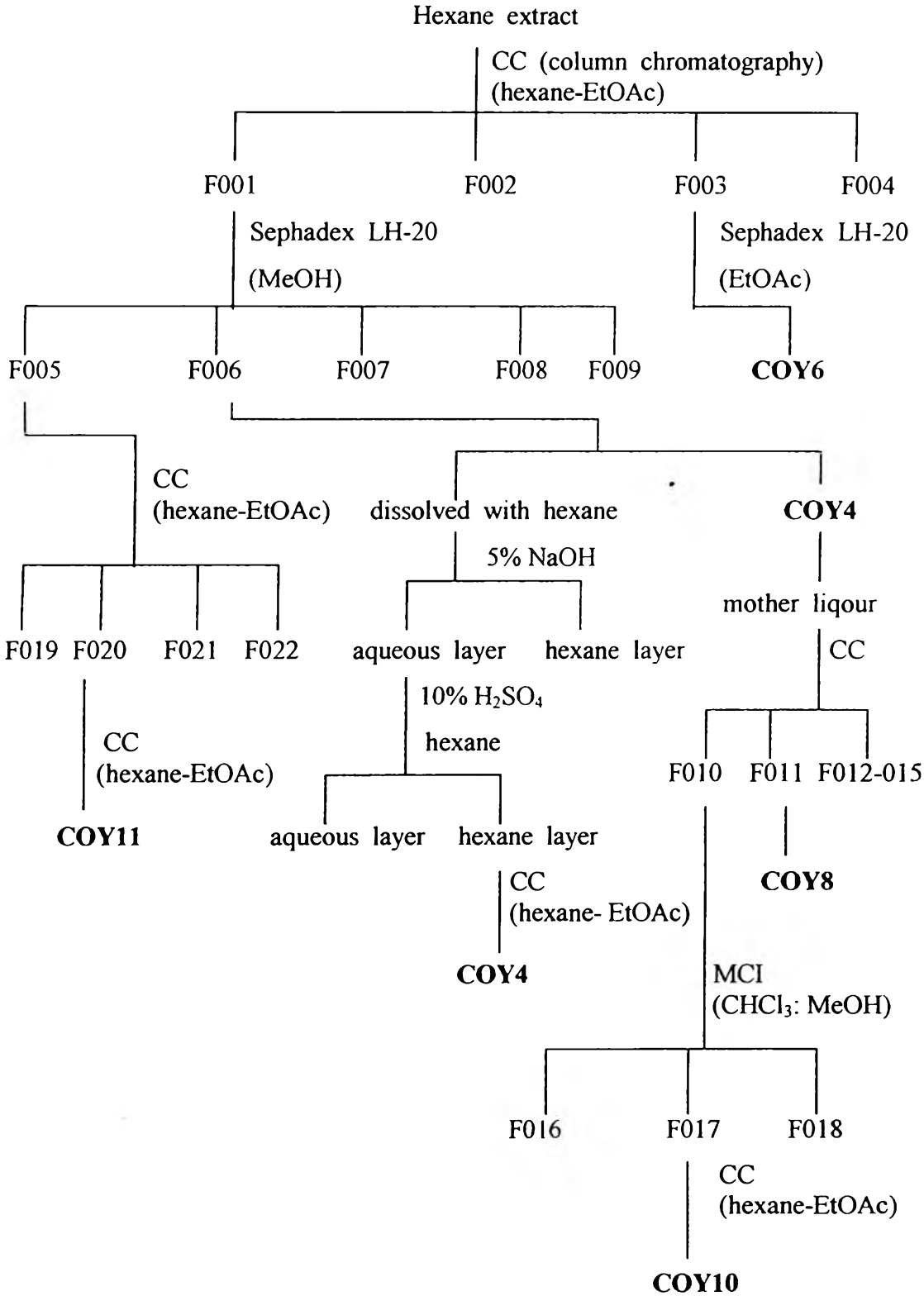
Fraction F017 was fractionated by column chromatography using silica gel 60 (No.9385) and gradient elution using hexane, hexane-EtOAc, EtOAc and $CHCl_3$. At 8% EtOAc in hexane, white needle crystals, COY10 (0.49 g, 0.21% w/w based on hexane extract) was obtained.

Isolation of compound COY11

Fraction F005 was separated by conventional chromatography using silica gel 60 (No.9385) and gradient elution using hexane and EtOAc. At concentration of 12% EtOAc in hexane, compound COY11 was obtained as white solid (0.06 g, 0.03%w/w based on hexane extract).

Isolation of isolate COY6

Fraction F003 was subjected to gel filtration chromatography using Sephadex LH-20 and EtOAc as eluent. The compound COY6 could be obtained as white needle like crystals (0.98 g, 0.43% w/w based on hexane extrac.).



Scheme 2 Isolation of hexane extract of *Croton oblongifolius*

Characterization of Isolated Compounds

1. Compound COY4

Compound COY4 was obtained as colorless crystals (6.23 g). It was soluble in $CHCl_3$, MeOH and hot hexane.

$[\alpha]_D^{20}$: -51.89° (c= 0.91, MeOH)

Melting point : 137-141°C

EI-MS : *m/z* (% relative intensity); Figure 2
302(12), 257(4), 175(37), 121(61), 81(72)

UV : λ_{max} nm (log ε), in MeOH
207(2.63)

FT-IR : ν_{max} cm^{-1}, KBr disc; Figure 3
3500-2500, 1703, 1646, 1598, 1391, 1277, 989, 900

^1H NMR : δ ppm, 500 MHz, in $CDCl_3$; Figures 4-5
6.79 (1H, dd, *J* = 6.7, 10.7), 5.31 (1H, dd, *J* = 6.7, 7.0), 5.19 (1H, d, *J* = 17.1), 5.09 (1H, d, *J* = 10.7), 4.84 (1H, s), 4.50 (1H, s), 2.42 (1H, dd, *J* = 6.5, 15.4), 2.35 (1H, ddd, *J* = 2.1, 4.3, 12.8), 2.19 (1H, ddd, *J* = 5.7, 11.4, 22.3), 2.08 (1H, dt, *J* = 3.6, 12.8), 1.99 (1H, dd, *J* = 2.9, 12.5), 1.82 (1H, partly overlap), ~1.80 (2H, partly overlap), 1.78 (3H, s), ~1.63 (1H, partly overlap), ~1.62 (2H, partly overlap), 1.48 (1H, dq, *J* = 4.3, 13.4), 1.36 (1H, dddd, *J* = 2.8, 5.3, 10.9, 21.1), 1.20 (1H, dt, *J* = 5.7, 12.2), 1.16 (3H, s), 0.76 (3H, s)

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; Figure 6
184.9(s), 147.8(s), 133.8(d), 131.8(d), 131.3(s), 113.3(t), 108.3(t), 57.2(d), 49.3(d), 47.5(s), 38.8(s), 38.1(t), 37.6(t), 37.1(t), 26.6(t), 21.9(t), 19.7(q), 18.4(t), 16.4(q), 14.7(q)

2. Compound COY11

Compound COY11 was obtained as white needle-like crystals (0.06 g). It was soluble in $CHCl_3$, MeOH and hexane.

$[\alpha]_D^{20}$: +27.23° (c= 0.22, MeOH)

Melting point : 83-86°C

EI-MS : m/z (% relative intensity); Figure 18
286(40), 271(7), 191(41), 95(100)

UV : λ_{max} nm (log ε), in MeOH
220.4(1.01), 201.7(1.15)

FT-IR : ν_{max} cm^{-1}, KBr disc; Figure 19
2931, 1644, 1513, 885, 720

^1H NMR : δ ppm, 500 MHz, in $CDCl_3$; Figures 20-22
7.16 (1H, d, *J* = 1.8), 6.10 (1H, d, *J* = 1.8), 4.74 (1H, q, *J* = 1.5), 4.55 (1H, q, *J* = 1.5), 2.71 (1H, dd, *J* = 3.4, 14.9), 2.60 (1H, dd, *J* = 10.4, 15.3), 2.33 (1H, ddd, *J* = 2.4, 4.2, 12.9), 2.28 (1H, dd, *J* = 3.5, 10.4), 2.00 (1H, dt, *J* = 5.2, 13.1), 1.95 (3H, s), 1.75 (1H, dtd, *J* = 1.8, 5.5, 14.3), 1.71 (1H, pd, *J* = 2.4, 12.5), 1.48 (1H, pd, *J* = 3.7, 14.3), 1.57 (1H, tq, *J* = 3.4, 13.6), 1.39 (1H, dtd, *J* = 1.5, 3.2, 13.1), 1.32 (1H, dq, *J* = 4.3, 2.8), 1.20 (1H, dt, *J* = 4.3, 14.0), 1.18 (1H, dd, *J* = 2.6, 12.7), 1.14 (1H, dt, *J* = 3.9, 12.8), 0.87 (3H, s), 0.81 (3H, s), 0.75 (3H, s)

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; Figure 23
150.9(s), 148.7(s), 139.3(d), 113(s), 112.8(d), 106.8(t), 55.4 (d), 54.4 (d), 42.1(t), 39.5(s), 38.9(t), 38.1(t), 33.6(q), 33.6 (s), 24.2(t), 21.8(q), 21.5(t), 19.4(t), 14.2(q), 10.1(q)

3. Compound COY10

Compound COY10 was obtained as white needle-like crystals (0.49 g). It was soluble in $CHCl_3$, MeOH and hot hexane.

$[\alpha]^{20}$: -79.15° (c= 0.74, MeOH)

Melting point : 177-179°C

EI-MS : m/z (% relative intensity); Figure 34
302(25), 287(26), 259(24), 241(27), 91(100)

UV : λ_{max} nm (log ε), in MeOH
203(0.90)

FT-IR : ν_{max} cm^{-1}, KBr disc; Figure 35
3400, 2927, 1693, 1468, 1264, 1179, 870

^1H NMR : δ ppm, 500 MHz, in $CDCl_3$; Figures 36-37
4.78 (1H, br s), 4.72 (1H, br s), 2.62 (1H, dd, *J* = 3.9, 4.3), 2.14 (1H, br d, *J* = 14.9), 2.03 (2H, s), 1.97 (1H, dd, *J* = 1.8, 11.3), 1.88 (1H, partly overlap), 1.86 (1H, overlap), 1.81 (1H, partly overlap), 1.6 (2H, overlap), 1.5 (2H, partly overlap), 1.45 (1H, partly overlap), 1.22 (3H, s), 1.11 (1H, dd, *J* = 4.9, 10.9), 1.06 (1H, overlap), 1.04 (1H, partly overlap), 0.98 (1H, overlap), ~0.93 (1H, partly overlap), 0.93 (3H, s), 0.8 (1H, partly overlap)

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; Figure 38
183.8(s), 155.9(s), 102.9(t), 57.1(d), 55.1(d), 48.9(t), 44.2 (d), 43.8(s), 43.7(s), 41.3(t), 40.7(t), 39.7(t), 39.66(s), 37.8(t), 33.1(t), 28.9(q), 21.8(t), 19.1(t), 18.4(t), 15.6(q)

4. Isolate COY6

Isolate COY6 was obtained as white needle like crystals (0.98 g). It was soluble in $CHCl_3$, MeOH and hot hexane.

Melting point : 149-151°c

EI-MS : m/z (% relative intensity); Figure 49
414(26), 412(34), 396(11), 381(7), 329(14), 300(18), 273(18), 271 (28), 255(40), 213(32), 159(63), 105(92) 83(95), 55(96)

UV : λ_{max} nm (log ε), in MeOH
203(0.82)

FT-IR : ν_{max} cm^{-1}, KBr disc; Figure 50
3600-3100, 2936, 1464, 1381, 1060, 970

^1H NMR : δ ppm, 300 MHz, in $CDCl_3$; Figure 51
5.35(1H,d), 5.10(1H,m), 3.5(1H,m), 2.35-0.6

GC : condition : column OV-1, detector FID,
column temperature 255°C, injection temperature 290°C, detector temperature 290°C, carrier gas N_2 40 ml/min.
Chromatogram showed 3 peaks at retention time 18.99, 20.21 and 22.93 min, respectively. Figure 52

5. Isolate COY8

Isolate COY8 was obtained as white needle crystals (0.06 g).Soluble in hexane, $CHCl_3$ and MeOH.

EI-MS : m/z (% relative intensity); Figure 53
382(9), 366(11), 357(5), 342(9), 335(14), 323(16), 319(24), 313 (16), 282(19), 265(29), 253(31), 241(39), 226(51), 211(100), 197 (52), 183 (76), 172(67), 158(59), 147(66), 133(88), 121(57), 107 (39), 93(41), 80(29), 69(18), 66(21), 57(21)

UV : λ_{max} nm (log ε), in MeOH
203(0.34)

FT-IR : ν_{max} cm^{-1} , KBr disc; Figure 54
3600-3000, 2938, 2866, 1734, 1691, 1463, 1379, 1245, 1027

GC : condition : column OV-1, detector FID,
column temperature 250°C, injection temperature 280°C, detector temperature 280°C, carrier gas N_2 40 ml/min.
Chromatogram showed peaks at retention time 2.18, 2.73, 3.44, 4.57, 5.60, 7.19, 9.30 and 12.08 min, respectively (Figure 56).