## CHAPTER II

## EXPERIMENTS

*General experimental procedures.* All solvents were distilled prior to use. UV-VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in EtOH. IR spectra were obtained on a Perkin Elmer Model 1760x Fourier Transform Infrared Spectrophotometer. Spectra of solid samples were recorded as KBr pellets and liquid samples were recorded as thin films (KBr cells). Low resolution mass spectra were obtained with a Fisons Instruments Mass Spectrometer model Trio 2000 at 70 ev. EIMS spectra were measured on a Bruker FTMS model CX47. $^{1}H$ and $^{13}C$ NMR spectra were recorded at 200.13 and 50.32 MHz, respectively,on a Bruker Model AC-F200 Spectrometer, and at 500.00 and 125.65 MHz on a JEOL JNM-A500 spectrometer in $CDCl_3$. Chemical shifts are given in parts per million using residual protonated solvent as reference . COSY, NOESY HMQC and HMBC experiments were performed on the JEOL JNM-A500 Spectrometer. Elemental Analysies were measured on a Perkin Elmer PE2400 SERIES II (CHN/O ANALYSER). Silica gel (Merck Kieselgel 60 and silica TLC plates (Si gel 60 $F_{254}$ ) were purchased from Merck Company.

*Plant material.*The plant material of *Croton oblongifolius* used in this study was collected from Ampur Pranburi Prachupkhirikhan province, Thailand in September 1996. The plant specimen was compared against voucher specimen no. BKF 084729 deposited in the herbarium of the Royal Forest Department of Thailand.

*Extraction and isolation.* The powdered, sun-dried stem barks (2.5kg) of *Croton oblongifolius* was repeatedly extracted with methanol. The methanol extract was filtered and evaporated under reduced pressure to obtain a dark-red gummy residue which was repeatedly reextracted with hexane, ethyl acetate and methanol respectively.

**Isolation of Crude Extract of *Croton oblongifolius* Roxb.**

**Separation of Hexane Crude Extract.**

The hexane crude extract was obtained as a yellowish green oil (90g) after evaporation. The crude hexane extract (40g) was fractionated by Silica gel column chromatography using Merck's silica gel Art.7734.1000 (70-230 mesh ASTM) as adsorbent. The column was eluted with hexane-ethyl acetate gradient in a stepwise fashion. The result of separation of hexane crude extract gave compounds 1-4 shown in table 1.

**Separation of Ethyl acetate Crude Extract.**

Concentrated ethyl acetate crude extract (20g) was separated on Silica gel 70-230 mesh ASTM using column chromatography technique. The column was eluted with hexane, hexane-ethyl acetate , ethyl acetate, ethyl acetate-methanol, respectively. The eluted fraction was collected about 500 ml and evaporated to about 30 ml to give gummy residues.

**Separation of Methanol Crude Extract.**

The methanol crude extract (6g) was purified with column chromatography on Sephadex LH-20 and the condition was eluted with $CHCl_3$-MeOH (1:1) about 20 ml of each fraction was collected. Similar fractions were combined on the basis of TLC analysis to result a semisolid fraction with small amount.

**Table 1** The results of separation of hexane crude extract by column chromatography.

| compounds | physical appearance | % wt by wt |
|---|---|---|
| 1 | transparent oil | 0.38 |
| 2 | white solid | 0.14 |
| 3 | white solid | 0..39 |
| 4 | white solid | 2.13 |

**Purification and Properties of the Compounds Eluted from Column Chromatography of Hexane Crude Extract.**

**Purification and Properties of Compound 1**

Compound 1 was eluted with pure hexane. The solvent was removed by rotary evaporation and the residue was purified by column chromatography (Merck's silica gel Art. 1.09385.1000 ). It is soluble in hexane, dichloromethane, chloroform, ethyl acetate ,diethyl ether and methanol.

Compound 1 is a transparent oil (4.25g, 0..38%), $[\alpha]_D^{30}$ +3.77 ($CHCl_3$, *c* 1.76), $R_f$ ; 0.83 (pure hexane), UV $\lambda_{max}$ (EtOH) 232sh ($\log\varepsilon$ 4.44), EA; Found C 88.12, H 11.62% Calc. C 88.23, H 11.76%.

FT-IR spectrum (neat) (Fig.22) $\nu_{max}$ ($cm^{-1}$) : 2924(h),2867(h),1672(w), 1645(w),1453(m),1384(m).

$^1$H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.23, table3) $\delta$ (ppm) : 6.35(1H,dd), 5.51(1H,t), 5.40(1H,dt), 5.04(1H,d), 4.80(1H,d), 2.29(1H,m),2.11(1H,m), 1.97(1H,d), 1.89(1H,m), 1.86(1H,m), 1.74(3H,s), 1.60(3H,s), 1.53(1H,m), 1.45(1H,m), 1.40(1 H,m), 1.19(1H,dd), 1.16(1H,dd), 1.00(1H,dt), 0.88(3H,s),0.86(3H,s), 0.79(3H,s).

$^{13}$C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.24, table3) $\delta$ (ppm) : 141.74(d), 135.91(d), 135.00(s), 132.42(s), 122.62(d), 109.79(t), 55.22(d), 50.20(d), 42.26(t),

39.74(t), 36.81(s), 33.27(q), 32.98(s), 26.28(t), 23.75(t), 22.46(q), 21.97(q), 18.89(t), 14.03(q), 11.85(q).

*m/z* (EI) (rel int.) Fig.26 : 272[$M^+$](4),191(99),135(19),121(31),109(100),95 (58),81(29),69(30),55(35) and 41(43).

**Purification and Properties of Compound 2**

Compound 2 was eluted with 5% ethyl acetate in hexane. Similar fraction were combined and the solvents were removed by rotary evaporation and futher purified by column chromatography (Merck's silica gel Art. 1.09385.1000 the column was eluted with 6% ether in hexane). This compound is soluble in hexane, dichloromethane, chloroform ,ethyl acetate ,diethyl ether and methanol.

Compound 2 is a white solid (1.60 g, 0.14%), $[\alpha]_D^{30}$ +37.48 ($CHCl_3$, *c* 1.51), $R_f$ ; 0.64 (30% chloroform in hexane), mp 72-74 °C, UV $\lambda_{max}$ (EtOH) 232sh ($\log\varepsilon$ 4.53), EA ; Found C 83.86, H 10.66% Calc. C 83.92, H 10.49%.

FT-IR spectrum (KBr) (Fig.31) $\nu_{max}$ ($cm^{-1}$) : 2954(h), 2923(h), 2852(h), 2710 (w), 1690(h), 1639(m), 1607(w), 1460(m), 1443(m), 1389(m), 1368(m).

$^1$H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.32, table7) $\delta$ (ppm) : 9.37(1H,s), 6.83(1H,dt), 6 29(1H,dd), 5.44(1H,t), 5.00(1Hd), 4.84(1H,d), 2.62(1H,m), 2.48(1 H,m), 2.34(1H,m), 2.30(1H,m), 2.18(1H,m), 1.90(1H,dd), 1.70(3H,s), 1.501H,m), 1.43(1H,d), 1.40(1H,d), 1.14(1H,d), 1.12(1H,d), 1.00(1H,dt), 0.91(3H,dd), 0.86(3H,s), 0.79(3H,s).

$^{13}$C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.33, table7) $\delta$ (ppm) : 194.31(d), 151.80(d), 143.40(s), 141.77(d), 133.77(d), 132.56(s), 109.79(t), 49.41(d), 49.38(d), 41.86(t), 39.87(t), 36.71(s), 33.22(q), 32.85(s), 24.90(t), 24.75(t), 22.07(q), 18.50(t), 14.73(q), 11.76(q).

*m/z* (EI) (rel int.) Fig.35 : 286[$M^+$](18),271(15),191(10),163(14),147(13),

124(24),109(100),91(38),81(62),55(22) and 41(25).

**Purification and Properties of Compound 3**

Compound 3 was eluted with 10% ethyl acetate in hexane. Similar fractions were combined and the solvents were removed by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000 the column was eluted with 60% dichloromethane in hexane). This compound is soluble in hexane, dichloromethane, chloroform ,ethyl acetate ,diethyl ether ,methanol.

Compound 3 was a white solid (4.29g, 0.39%), $[\alpha]_D^{30}$ +12.02 ($CHCl_3$, *c* 1.63), $R_f$ ; 0.77 (pure chloroform), mp 90-92 °C, UV $\lambda_{max}$ (EtOH) 234sh (log$\varepsilon$ 4.54), EA; Found C 83.19, H 11.28% Calc. C 83.33, H 11.11%.

FT-IR spectrum (KBr) (Fig.40) $\nu_{max}$ ($cm^{-1}$) : 3249(Br,s),2919(s),2845(s), 1640(w),1607(w),1459(m),1441(m),1388(m),1365(m),1073(m),1057(m).

$^1$H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.41, table10) $\delta$ (ppm) : 6.34(1H,dd), 5.75(1H,dt),5.55(1H,t),5.06(1H,d),4.91(1H,d),4.05(1H,d),3.86(1H,d),2.33(1H,d),2.15 (1H,m),2.05(2H,m), 1.90(2H,m), 1.77(3H,s), 1.45(1H,m), 1.01(1H,m),0.87(3H,s),0.85 (3H,s),0.77(3H,s).

$^{13}$C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.42, table10) $\delta$ (ppm) : 141.27(d), 138.57(s),134.70(d),133.54(s),125.40(d),110.67(t),65.98(t),52.23(d),49.84(d),42.15(t), 39.57(t),36.66(s),33.19(q),32.96(s),25.85(t),23.55(t),21.95(q),18.83(t),13.99(q),11.94 (q).

*m/z* (EI) (rel int.) Fig.44 : 288[$M^+$](3),270(19),255(15),202(12)189(21),176 (20),161(16),147(25),131(52),109(100),105(69),91(84),81(92),55(76) and 41(96).

**Purification and Properties of Compound 4**

Compound 4 was obtained from 10% ethyl acetate in hexane fractions on silica gel column chromatography. The compound was recrystallized from hexane for several times to provide the colourless needle-like crystals (23.64g, 0.96% wt by wt. of hexane crude) with a m.p.118-120°C. This compound is soluble in chloroform, ether, ethyl acetate, methanol and hot hexane.

Compound 4 was colourless crystals (23.64g, 2.13%),$[\alpha]_D^{30}$ -15.93 ($CHCl_3$, *c* 1.67), $R_f$ ; 0.29 (pure chloroform), mp 118-120 °C, UV $\lambda_{max}$ (EtOH) 232sh (log$\varepsilon$ 4.47),EA; Found C 79.49, H 9.66% Calc. C 79.47, H 9.93%.

FT-IR spectrum (KBr) (Fig.49) $\nu_{max}$ ($cm^{-1}$) : 3421-2627(Br,s),2946(s),1707(s), 1652(s), 1604(w),1459(w),1430(m),1383(m),1345(w),1208(s).

$^1$H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.50, table13) $\delta$ (ppm) :6.90(1H, td),6.31(1H,dd),5.47(1H,t),5.0(1H,d),4.84(1H,d),2.58(1H,m),2.37(1H,m),2.32(1H,dd), 2.19(1H,m),1.87(1H,d),1.67(3H,s),1.40(2H,m),1.18(1H,m),0.89(3H,s),0.86(3H,s),0.82 (3H,s).

$^{13}$C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig.51, table13) $\delta$ (ppm) : 174.9(s), 141.77(d),140.52(d)133.64(s),133.44(d)133.08(s),109.94(t),49.95(d),49.34(d),41.93 (t),40.08(t),36.88(s),33.31(q),32.8(s),26.03(t),23.98(t),22.15(q),18.57(t),14.78(q), 11.72(q).

*m/z* (EI) (rel int.) Fig.53 : 302[$M^+$](50),284(14),221(47),203(44),175(65),151 (67),139(100),125(80),109(95),81(94),69(68),55(64) and 41(67).

*Methylation of compound 4 (5).* The compound 4 (100 mg, 0.33 mmol) was methylated with diazomethane in ether under the conditions described earlier [13], and gave compound 5 as a viscous oil (100.45 mg, 96%), $[\alpha]_D^{30}$ -11.93 ($CHCl_3$, *c*0.97), $R_f$ ; 0.79 (50% chloroform in hexane), UV $\lambda_{max}$ (EtOH) 230sh (log$\varepsilon$ 4.17).

FT-IR spectrum (neat) $\nu_{max}$ ($cm^{-1}$) : 952(s),1730(s),1657(w),1610(w),1466(w), 1437(w),1388(m),1354(w),1267(s),1070(s).

$^1$H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.58) $\delta$ (ppm) : 6.62(1H,dt),5.42(1 H,t),5.01(1H,d),4.85(1H,d),3.59(3H,s),2.50(1H,m),2.38(1H,m),2.22(1H,m),2.10(1 H,dt),1.87(1H,dd),1.47(1H,m),1.43(1H,m),1.38(1H,m),1.27(1H,d),1.68(3H,s),1.14(1 H,d),1.03(1H,dd),0.88(3H,s),0.85(3H,s) and 0.81(3H,s).

$^{13}$C-NMR spectrum ($CDCl_3$, 50 MHz) (Fig.58, table15) $\delta$ (ppm) : 169.54(s), 141.72(d),137.04(d),134.52(s),133.62(d),133.06(s),109.96(t),51.32(q),50.33(d),50.32 (d),41.95(t),39.92(t),36.78(s),33.28(s),32.80(q),26.23(t),23.71(t),22.13(q),18.57(t), 14.51(q) and 11.73(q).

*m/z* (EI MS) (rel int.) Fig.59 : 316[$M^+$](46),284(50),235(42),203(70), 175(96),165(56),153(80),139(82),133(55),119(67),190(100),105(71),93(53),91(75),81 (79),79(68),69(59),55(68) and 41(59).

*Reduction of Compound 5.* Methyl ester (80 mg, 0.5 mmol) in 10 ml of anhydrous diethyl ether was added slowly from a dropping funnel into a stirred solution of lithium aluminium hydride (62.37 mg, 1.64 mmol) in 20 ml of anhydrous diethyl ether in a 50 ml round-bottom flask previously flushed with nitrogen. After the addition was completed, the reaction mixture was stirred for 5 hours at room temperature. The reaction was stopped and worked up by a usual manner. The organic layer was concentrated by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000) and eluted with 30% ether in hexane to result compound 3 (58.32 mg, 80%). The spectral data ($^1$H and $^{13}$C-NMR, IR and MS) of this compound was identical to that of naturally occurring.