CHAPTER III

EXPERIMENTAL

1. Source and Authentication of Plant Materials

The bark of *Albizia myriophylla* Benth. were collected from Chachoengsao province, Thailand in September 1996. The plant materials were identified by comparison with the herbarium specimen in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand.

2. General Techniques

2.1. Thin Layer Chromatography (TLC)

Technique	: one way, ascending
Adsorbent	: silica gel G (E. Merck), 30 gm / 60 ml of distilled water
Plate size	: 20 cm x 20 cm , 20 cm x 10 cm
Layer thickness	: 0.2 mm
Activation	: air dried for 15 minutes and then at 110 $^{\circ}$ C for 1 hour.
Solvent system	: silica gel G / chloroform, methanol , strong ammonium hydroxide solution (9:1:0.5)
Distance	: 15 cm
Laboratory temperature	: 25 - 30 $^{\circ}$ C
Detection	: 1. The spot gave an orange red colour with Dragendorff's spray reagent 2. The spot gave an brown colour with iodine vapour

3. Visual detection under ultraviolet light at the wavelength of 254 and 365 nm.

2.2. Column Chromatography

Adsorbent : silica gel 60 (No. 7734) particle size 0.063-0.200 mm
silica gel 60 (No. 9385) particle size 0.040 - 0.063 mm. (E. Merck)

Packing : dry packing

Adding of a crude extract to column : A crude extract was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent,
air dried, triturated and added to the top of a dry column.

Solvent : a) chloroform : methanol : strong ammonium hydroxide solution (9:1:0.5)
b) chloroform : methanol : strong ammonium hydroxide solution (8:2:0.5)
c) chloroform : methanol (9:1)

Collection of eluate : fraction of 50 ml

Examination of eluate : Those fractions giving an orange colour with Dragendorff's spray reagent were examined by thin layer chromatography and giving pink-red colour with Liebermann-Burchard test.

2.3. Physical Property

Melting Point Determination

All melting points were determined by Gallenkamp melting point Apparatus in the Department of Pharmacognosy, the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4 Spectroscopy

2.4.1 Nuclear Magnetic Resonance (NMR) Spectra

The proton (^{1}H) and carbon-13 (^{13}C) NMR spectra were taken on a Jeol alpha FT NMR spectometer operated at 300 and 500 MHz proton frequency (Faculty of Pharmaceutical Sciences, Chiba University) with tetramethylsilane (TMS) as internal standards. The multiplicities for ^{13}C NMR spectra were determined by Distortionless Enhancement by Polarization Transfer (DEPT).

The solvent for NMR spectra was deuterated chloroform ($CDCl_3$) and 10% deuterated methanol.(CD_3OD)

2.4.2 Mass Spectra

The mass spectra were recorded by Jeol model JMS-DX 300 at 70 eV and ionization current 300 μA, in the Scientific and Technological Research Equipment Center, Chulalongkorn University. and JEOL model JMS-DX 700 using fast atom bombartment (FAB) technique.

3. Extraction

The dried coarsely powdered barks (4.0 kg) were macerated with methanol (15 L) for three days and filtered. The marc was remacerated with methanol (15 L) for three days and filtered. The filtrates were concentrated under reduced pressure and combined to yield the total crude extract. The total crude extract was dissolved in methanol (100 ml) and then poured into distilled water (200 ml) to give the solution. This solution was extracted with chloroform (6x500 ml). The combined

chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure to dryness to give a chloroform extract (42.1 g). The chloroform extract contained at least four alkaloids, as shown in thin layer chromatography on silica gel plate (Figure 9 , page 88)

4. Isolation

The crude chloroform extract (42.1 g) was divided into 4 portions and each portion was treated in the same manner. Each portion (approx 10 g) was purified by silica gel column. (2.5x15 cm.) Using chloroform:methanol:strong ammonium hydroxide (9:1:0.5) as the eluent fifty milliliters fractions were collected and examined by thin layer chromatography (TLC). Those fraction of similar pattern were combined and evaporated to dryness as following :

Fraction 1 - 10 afforded a residue impurity

Fraction 10 - 30 afforded a residue A

Fraction 30 - 40 afforded a residue B

Fraction 40 - 60 afforded a residue C

4.1 The Isolation of AM-1

Residue A was further purified using a column of Sephadex LH-20 (2x70) with methanol as eluent the 10 fraction eluated was collected depending on color band. (approximately 20 ml per fraction). A yellow syrup mass (fraction 4-7) rechromatographed on silica gel (2.5x15 cm) column. Using the same as above process and collected the fraction that has the alkaloid AM-1 as major compound combined together and evaporated to dryness, dissolved in small amount of chloroform and ethyl acetate was added dropwise to yield the white needle crystals (approx 600 mg), designated as AM-1

4.2 The Isolation of AM-3

Residue B was further purified using a column of Sephadex LH-20 (2x70) with methanol as eluent the 10 fraction eluated was collected depending on color band. A yellow oil syrup mass was rechromatographed on silica gel (2.5x15 cm) column. Using chloroform : methanol : concentrated ammonium hydroxide (8:2:0.5) as the eluent fifty milliliters fractions were collected and examined by thin layer chromatography. Fraction 10 - 15 were designated as AM-3 as minor alkaloid compound

4.3 The Isolation of TS-1

Residue C combined together and evaporated under reduced pressure to dryness yield brown mass. The brown mass was rechromatographed on silica gel (2.5x15 cm) column. Using chloroform : methanol (9:1) as the eluent fifty milliliters fractions were collected and examined by thin layer chromatography. Fraction 15-25 were designated as TS-1. (colorless needles 15 mg.)

5. Characterization of the Isolated Compounds

5.1 Characterization of AM-1

AM-1 was obtained as white needle crystal. It was soluble in chloroform.

EIMS ; *m/z* (% relative intensity) ; Figure 10

310 (100), 250(7), 208(4), 176(7), 154(27), 95(17), 41(43), 29(21), 23(15)

^{1}H NMR ; δ ppm, 500 MHz($CDCl_3$/TMS) ; Figure 11-12

3.88 (d, J = 15 Hz), 4.12 (m), 4.65 (br s), 5.63 (t, J = 10 Hz), 5.73 (t, J = 10 Hz), 6.83 (d, J = 10 Hz)

5.2 Characterization of AM-3

AM-3 was obtained as yellow oil. It was soluble in chloroform.

EIMS ; *m/z* (% relative intensity) ; Figure 13

308 (100), 250(3), 217(4), 176(22), 136(19), 91(14), 55(22), 41(21), 23(19)

^{1}H NMR ; δ ppm, 500 MHz($CDCl_3$/TMS) ; Figure 14-15

1.10 (t , J = 15 Hz), 1.25 (m), 1.58 (m), 6.75 (t, J = 10 Hz)

5.3 Characterization of TS-1

TS-1 was obtained as colorless needles crystal. It was slightly in chloroform.

EIMS ; *m/z* (% relative intensity) ; Figure 16

454 (7), 245(100), 231(4), 218(9), 207(20), 190(25), 159(5), 131(10), 107(10), 81(11), 69(15), 54(21)

^{1}H NMR ; δ ppm, 300 MHz ($CDCl_3$/TMS) ; Figure 17-19

0.78 (br s) 0.8 (br, s) 0.9 (br, s), 1.0 (br s), 1.2 (br s), 1.6(m), 2.14 (m), 3.38 (m), 4.18 (dd, J = 6 Hz), 5.31 (t, J = 3 Hz), 7.3 (s)

^{13}C NMR; δ ppm, 300 MHz ($CDCl_3$, CD_3OD 10 %,) ; Figure 20-24

15.4, 15.4, 16.7, 18.1, 20.6, 23.3, 23.7, 24.2, 24.7, 25.0, 26.6, 27.8, 32.9, 33.2, 35.0, 36.85, 38.4, 38.5, 39.1, 39.5, 39.6, 42.3, 43.1, 47.3, 55.0, 78.5, 83.3, 124.6, 139.9, 182.9