

CHAPTER III

EXPERIMENTAL

1. Sources of plant material

The roots of *Alangium salviifolium* Wang. subsp. *hexapetalum* Wang. were collected in September 1994 from Karnchanaburi province, Thailand. The plant material was authenticated by comparison with the herbarium specimen at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative.

The leaves of this plant were collected from the plant growing in the open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University in October 1995.

2. General Techniques

2.1 Thin-layer Chromatography (TLC)

Technique	: One way, ascending.
Adsorbent	: Silica gel 60 F 254 (E. Merck) precoated plate
Layer thickness	: 0.2 mm
Solvent system	: Dichloromethane : methanol : ammonia (90:9:1)
Distance	: 5 cm
Temperature	: Laboratory temperature (30-35°C)
Detection	: 1. Ultraviolet light at the wavelength of 254 and 365 nm : 2. Dragendorff's spraying reagent

2.2 Preparative Thin-layer Chromatography

Technique	: One way, ascending.
Adsorbent	: Precoated for preparative layer chromatography plate

	Silica gel 60 GF 254 (E. Merck)
Layer thickness	: 1 mm
Solvent system	: Dichloromethane : methanol : ammonia (90:9:1)
Distance	: 20 cm
Temperature	: Laboratory temperature (30-35°C)
Detection	: 1. Ultraviolet light at the wavelengths of 254 and 365 nm : 2. Dragendorff's spraying reagent

2.3 Column Chromatography

2.3.1 Quick Column Chromatography

Column size	: 7 cm-diameter sintered glass column
Adsorbent	: Silica gel 60 (No.9385) particle size 0.040-0.063 mm (230-400 mesh ASTM) (E.Merck)
Packing method	: Dry packing
Sample loading	: Crude extract was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, then dried and added gently on the top of the column.
Eluents	: Dichloromethane : methanol : ammonia (97:2:1) Dichloromethane : methanol : ammonia (95:4:1) Dichloromethane : methanol : ammonia (93:6:1) Dichloromethane : methanol : ammonia (91:8:1)
Examination of eluates	: Fractions were separated by TLC and observed under ultraviolet light at the wavelengths of 254 and 365 nm and after spraying with Dragendorff's reagent.

2.3.2 Open Column Chromatography

Column size	: Glass column 5 x 15 cm
Adsorbent	: Silica gel 60 (No.9385) particle size 0.040-0.063 mm (230-400 mesh ASTM) (E.Merck)
Packing method	: Wet packing
Sample loading	: Sample was dissolved in a small amount of organic solvent

and added gently on the top of the column.

- Eluent** : Dichloromethane : methanol : ammonia (96:3:1)
- Examination of eluates** : Fractions were separated by TLC and observed under ultraviolet light at the wavelengths of 254 and 365 nm and after spraying with Dragendorff's reagent.

2.3.3 Medium Pressure Liquid Chromatography

- Equipment** : The columns were Buchi glass columns and the pump was Buchi 681 chromatography pump.
- Column sizes** : Glass column 2.5 x 24 cm and 2.5 x 20 cm
- Adsorbent** : Silica gel 60 (No.9385) particle size 0.040-0.063 nm (230-400 mesh ASTM) (E.Merck)
- Packing method** : Dry packing
- Sample loading** : Sample was dissolved in a small amount of organic solvent and loaded directly to the column.
- Eluents** : Dichloromethane : methanol : ammonia (96:3:1) for AL-1
Dichloromethane : methanol : ammonia (96:3:1),
Dichloromethane : methanol : ammonia (95:4:1) and
Dichloromethane : methanol : ammonia (94:5:1) for AL-2
Dichloromethane : methanol : ammonia (96:3:1) for AL-3
Dichloromethane : methanol : ammonia (93:6:1) for AL-4
Dichloromethane : methanol : ammonia (96:3:1) for AL-1L
- Pressure** : Less than 5 bars
- Examination of eluates** : Fractions were separated by TLC and observed under ultraviolet light at the wavelengths of 254 and 365 nm and after spraying with Dragendorff's reagent.

2.3.4 Gel Filtration Chromatography

- Column size** : Glass column 2 x 70 cm

- Adsorbent** : Sephadex LH-20 (Pharmacia)
- Packing method** : The material was suspended in the eluent and left standing to swell for 24 hours before pouring into the column and allowed to settle under gravity.
- Sample loading** : Sample was dissolved in a small volume of eluent and loaded onto the top of the column.
- Eluent** : Chloroform : methanol (9 : 1)
- Examination of eluates** : Fractions were separated by TLC and observed under ultraviolet light at the wavelengths of 254 and 365 nm and after spraying with Dragendorff's reagent.

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) Absorption Spectra

The uv spectra were recorded on a Shimadzu UV-240 spectrophotometer (Kobe Pharmaceutical University).

2.4.2 Infrared (IR) Absorption Spectra

The spectra were obtained from a Perkin Elmer FT-IR spectrometer 1760x (Scientific and Technological Research Equipment Center, Chulalongkorn University) in potassium bromide disc.

2.4.3 Mass Spectra (MS)

The mass spectra were obtained from a Hitachi M-4100 mass spectrometer (Kobe Pharmaceutical University).

2.4.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^1H and ^{13}C NMR) Spectra

The NMR experiments for AL-1, AL-3, AL-4 and AL-1L were performed on Varian VXR-500 and Varian Gemini-300 spectrometers (Kobe Pharmaceutical University).

The NMR experiments for AL-2 was performed on a JEOL JMN-A 500 spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

The Chemical shifts were reported in ppm scale using the chemical shift of tetramethylsilane (TMS) at 0 ppm as the reference signal.

2.5 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

2.6 Dragendorff's Spraying Reagent

Dragendorff's reagent was used as a general alkaloid detecting reagent. The alkaloids give orange spots as positive test after spraying.

Preparation of Dragendorff's reagent:

Solution A:

Bismuth subnitrate	850 mg
Distilled water	40 ml
Acetic acid	10 ml

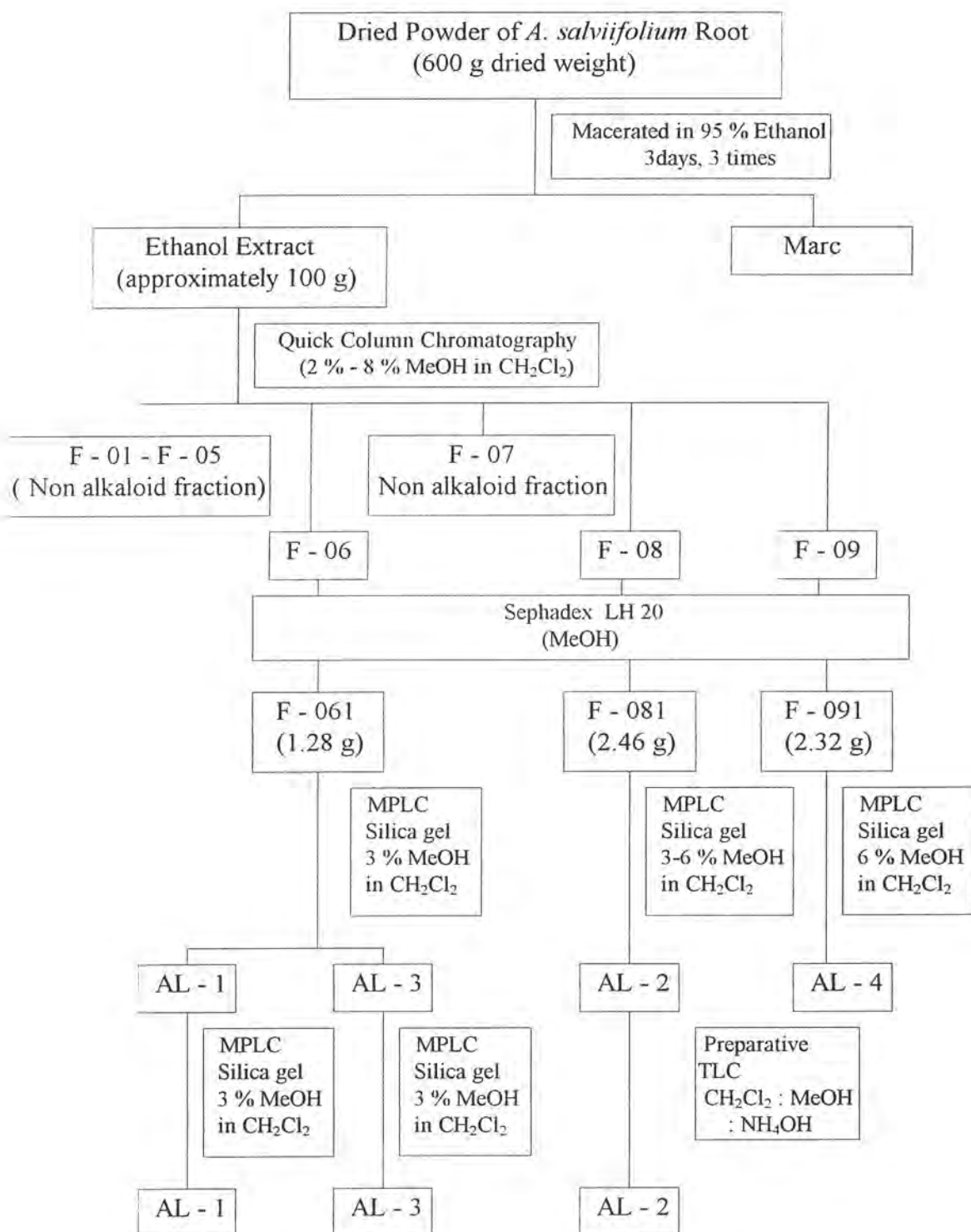
Solution B:

Potassium iodide	8 g
Distilled water	20 ml

Before use, 5 ml each of Solution A and Solution B were mixed, then 20 ml of glacial acetic acid and 70 ml of distilled water were added.

3. Extraction and Isolation of Alkaloids from *A. salviifolium* Root

The extraction and isolation procedure is shown in Scheme 7.



Scheme 7 Extraction and isolation scheme of *A. salviifolium* Wang. root

3.1 Extraction Procedure

The dried powder of *A. salviifolium* root (0.6 kg) was repeatedly macerated for a three 3-day periods in 95% ethanol. The ethanolic extract was then filtered. The filtrate from each maceration was concentrated under reduced pressure at temperature not exceeding 40°C. The obtained crude ethanolic extract was approximately 100 g and TLC analysis revealed the presence of at least 3 alkaloids.

3.2 Isolation Procedure

The crude extract (approximately 50 g) was dissolved in a small volume of chloroform and triturated with sufficient quantity of silica gel 60 (No.9385) and dried under vacuum. It was then fractionated by quick column chromatography using a sintered glass filter column of silica gel (7x3.5 cm). The column was eluted with a stepwise gradient from 2% to 8% methanol in dichloromethane. The eluates were examined by TLC using the solvent system as mentioned before. Fractions with similar chromatographic pattern were combined as shown in Table 3.

Table 3 Combined fractions from crude ethanolic extract of *A. salviifolium* roots

Fractions	Number of eluates	Weight (g)	Eluent (% methanol in dichloromethane)
F-01	1-19	1.88	2
F-02	20-23	1.16	2
F-03	34-54	0.32	4
F-04	55-75	1.37	4
F-05	76-86	0.21	4
F-06	87-129	2.68	6
F-07	130-140	1.50	6
F-08	141-162	4.23	8
F-09	163-173	4.43	8

By TLC analysis, various spots of alkaloid could be detected in fractions F-06, F-08 and F-09. Each of these combined fractions was purified by column chromatography using a Sephadex LH 20 column (2 x 70 cm) and the eluent was methanol. The eluates were collected into fractions (20 ml each) which were examined by TLC. From this step the alkaloid fractions F-061, F-081 and F-091 were obtained in quantity of 1.28 g, 2.46 g, and 2.32 g respectively.

3.2.1 Isolation of Alkaloids AL-1 and AL-3

Based on TLC, fraction F-061 (1.28 g) appeared to contain two main alkaloids. This fraction was divided into 400 mg per portion and purified by MPLC using the column of silica gel (2.5 x 24 cm) and the eluent of dichloromethane : methanol : ammonium hydroxide (96:3:1). The eluates were collected (15 ml per fraction) and, by TLC analysis, similar alkaloid-containing fractions were combined.

Two alkaloids were separated and codenamed AL-3 and AL-1. The alkaloid AL-3 was further purified by MPLC using a silica gel column (2.5 x 20 cm). The column was eluted with the same solvent mixture as described above. After evaporation to dryness, hot ethyl acetate was added. When left at room temperature, a yellow compound was obtained as amorphous powder. Its amount was 12.3 mg (2.0×10^{-3} % yield) and was subsequently identified as tubulosine.

The alkaloid AL-1 was purified by MPLC using a silica gel column (2.5 x 20 cm). The column was eluted with the same solvent mixture. After evaporation to dryness, hot dichloromethane was added. When left at room temperature, a yellow compound was obtained as 9.3 mg of amorphous powder (1.5×10^{-3} % yield). The alkaloid AL-1 was subsequently identified as psychotrine.

3.2.2 Isolation of Alkaloid AL-2

By TLC analysis, one main alkaloid was detected in fraction F-081 (2.46 g). This fraction was divided into 400-mg per portions and purified by

MPLC using a silica gel column (2.5 x 24 cm). The column was eluted with a stepwise gradient system from 3% to 5% methanol in dichloromethane containing 1% ammonium hydroxide. The alkaloid was detected in the fractions containing 4% methanol and was further purified by preparative TLC using dichloromethane: methanol : ammonium hydroxide (90:9:1) as the developing solvent. A yellowish white amorphous compound was obtained and was codenamed as alkaloid AL-2 (17.6 mg, 2.9×10^{-3} % yield). It was subsequently identified as emetine.

3.2.3 Isolation of Alkaloid AL-4

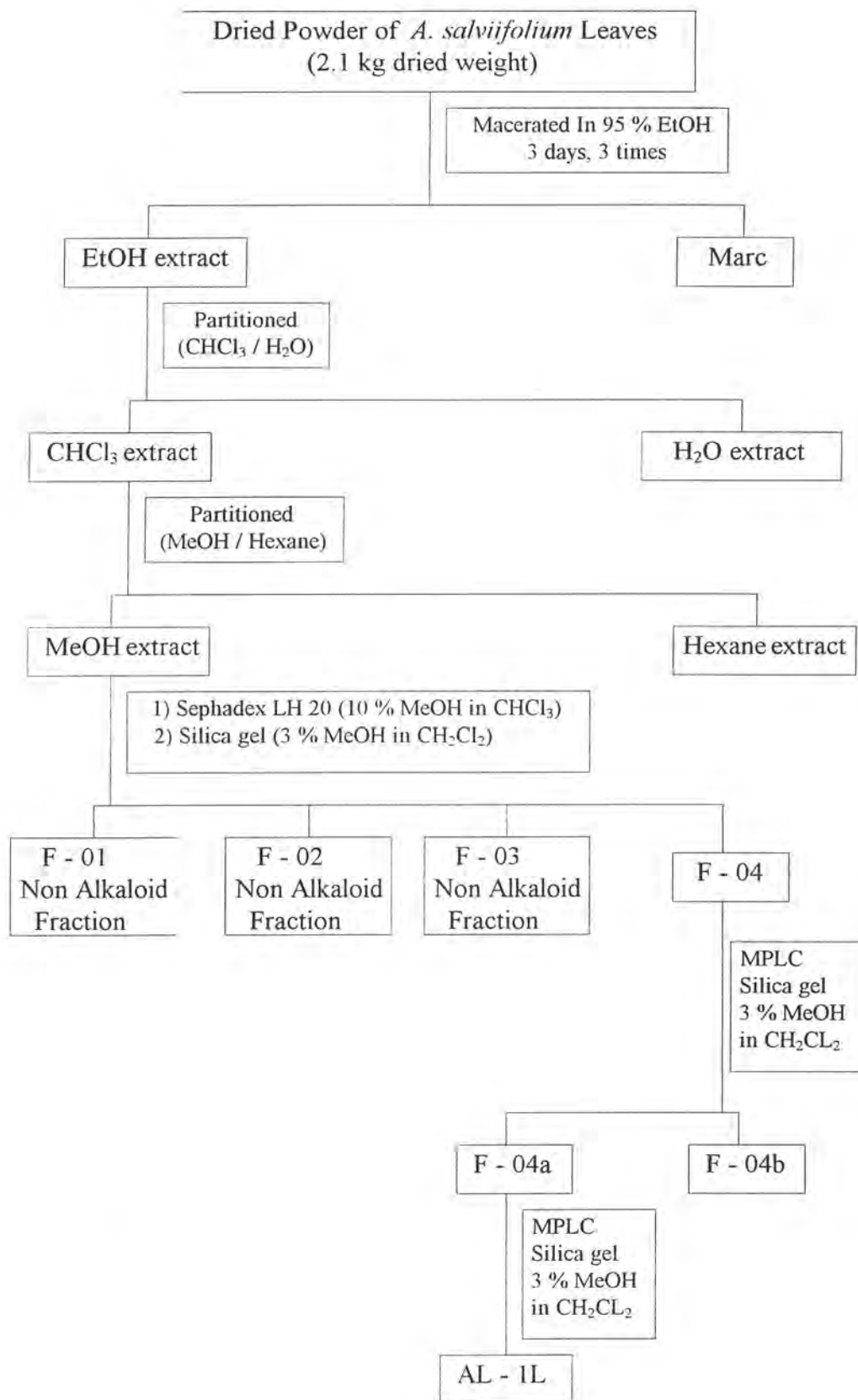
By TLC analysis, one main alkaloid was detected in fraction F-091 (2.32 g). The main alkaloid was isolated by MPLC using a silica gel column (2.5 x 24 cm) with 6% methanol in dichloromethane containing 1% ammonium hydroxide as the eluent. The isolated alkaloid was assigned the name AL-4 (11.4 mg, 1.9×10^{-3} % yield) and subsequently identified as demethylpsychotrine.

4. Extraction and Isolation of Alkaloids from *A. salviifolium* Leaves

The extraction and isolation procedure is shown in Scheme 8.

4.1 Extraction Procedure

The 2.1 kg dried powder of *A. salviifolium* leaves was repeatedly macerated with 95% ethanol for three 3-day periods and the extract was filtered. The filtrate from each maceration were pooled and concentrated under reduced pressure at temperature not exceeding 45°C to yield crude ethanolic extract (411.03 g). It was partitioned between chloroform and deionized water. By TLC analysis, alkaloids were detected in chloroform fraction. The chloroform fraction was evaporated to dryness under reduced pressure to give 106.05 g of syrupy mass. It was further partitioned between methanol and hexane. By TLC analysis, the alkaloids were detected only in the methanol fraction. It was evaporated to dryness under reduced pressure to give 80.30 g of syrupy mass.



Scheme 8 Extraction and isolation scheme of *A. salviifolium* Wáng. leaves

The chlorophylls were separated from the alkaloids by gel filtration chromatography using a column of Sephadex LH-20 (2 x 70 cm). The syrupy mass (80.30 g) was dissolved in 10% methanol in chloroform and was divided into 5 portions. Each portion was loaded on the column and eluted with 10% methanol in chloroform. The eluates, approximately 20 ml each, were collected and examined by TLC. Only the first four fractions contained alkaloids. They were combined and evaporated to dryness under reduced pressure to yield 56.67 g of alkaloid fraction.

4.2 Isolation Procedure

The alkaloid-containing part (56.67 g) was divided into 2 equal portions. Each one was fractionated by open column chromatography using a silica gel column (5 x 15cm) and 3% methanol in dichloromethane containing 1% ammonium hydroxide as the eluent. Twenty-millilitre fractions were collected and combined after examining with TLC, followed by spraying with Dragendorff's reagent. Fractions with similar chromatographic pattern were combined to give 4 fractions: F-01 (2.68 g), F-02 (2.34g), F-03 (1.92g) and F-04 (2.26g). After fraction F-04, no alkaloid was detected. Fraction F-04 was selected for further purification since alkaloids were detected in this fraction.

4.2.1 Isolation of Alkaloid AL-1L

One main alkaloid spot was detected in fraction F-04. It was purified by MPLC using a silica gel column. Elution of the column with 3% methanol in dichloromethane containing 1% ammonium hydroxide solution gave fractions 4a and 4b. Fraction 4a contained the alkaloid and was rechromatographed by the same technique to yield yellow amorphous powder. It was recrystallized from hexane as pale yellow needles and codenamed as AL-1L (22.3 mg, 1.0×10^{-3} % yield). It was later identified as a mixture of alangimarckine and 1'-dehydroalangimarckine.

5. Characterization of Isolated Compounds

5.1 Characterization of AL-1

AL-1 was obtained as yellow amorphous powder. It was soluble in chloroform and methanol.

EIMS	: m/z (% relative intensity) : Figure 2 (Page 50) 464 (15.5), 273 (59.2), 244 (100), 216 (8.2), 191 (13.9), 28 (9)
IR	: ν cm^{-1} : KBr disc : Figure 3 (Page 51) 3430, 2955, 1512, 1435, 1324, 1230, 1207
UV	: λ_{max} nm (log ϵ) in methanol : Figure 4 (Page 52) 207 (3.59), 277 (3.01), 406 (3.20)
^1H NMR	: δ ppm, 300 MHz, in CDCl_3 : Figure 5,6,7 (Page 55-57) 0.97 (3H, t, $J= 9.60$ Hz), 3.72 (3H, s), 3.8 (3H, s), 3.89 (3H, s), 6.45 (1H, s), 6.53 (1H, s), 6.76 (1H, s), 6.98 (1H, s)
^{13}C NMR	: δ ppm, 125 MHz, in DMSO-d_6 : Figure 8, 9 (Page 58-59) 10.9, 22.9, 25.7, 28.6, 37.2, 38.4, 40.0, 41.7, 48.4, 51.6, 55.0, 55.1, 55.3, 60.5, 61.9, 108.1, 109.9, 111.7, 114.1, 117.4, 126.3, 129.8, 132.5, 146.0, 146.5, 146.8, 153.8, 165.4

5.2 Characterization of AL-2

AL-2 was obtained as amorphous powder. It was soluble in chloroform and methanol.

EIMS	: m/z (% relative intensity) : Figure 10 (Page 62) 480 (87.5), 289 (87.5), 260 (100.0), 240 (15.3), 216 (14.5), 192 (50.0)
IR	: ν cm^{-1} : KBr disc : Figure 11 (Page 63) 3430, 3393, 3375, 3355, 2955, 2915, 1614, 1517
^1H NMR	: δ ppm, 500 MHz, in CDCl_3 : Figure 12, 13 (Page 65-66) 0.90 (3H, t, $J= 7.33$ Hz), 3.80 (3H, s), 3.81 (3H, s), 3.83 (3H, s), 3.86 (3H, s)

¹³C NMR : δ ppm, 125 MHz, in CDCl₃ : Figure 14, 15, 16
(Page 67-69)
10.9, 25.1, 22.5, 25.9, 33.5, 34.7, 38.0, 39.2, 40.6, 51.2, 55.8, 55.9,
56.3, 56.7 (x4), 109.1, 109.8, 111.4, 110.9, 123.7 (x3), 148.1, 148.4,
148.6, 148.7

5.3 Characterization of AL-3

AL-3 was obtained as pale yellow amorphous powder. It was soluble in chloroform and methanol.

EIMS : m/z (% relative intensity) : Figure 17 (Page 72)
475 (100), 445 (9.4), 272 (48.4), 246 (41.7), 205 (19.0), 187 (52.0)

IR : ν cm⁻¹ : KBr disc : Figure 18 (Page 73)
3381, 2937, 2909, 2847, 1,512, 1463, 1251, 1229, 1212

UV : λ_{\max} nm (log ϵ) in methanol : Figure 19 (Page 74)
205 (3.35), 281 (2.71)

¹H NMR : δ ppm, 500 MHz., in DMSO-d₆ : Figure 20, 21, 22, 23 (Page 77-80)
0.86 (3H, t, J = 6 Hz), 3.690 (3H, s), 3.698 (3H, s), 4.1 (1H, br d,
 J = 8.4 Hz) 6.46 (1H, dd, J = 6.8, 1.6 Hz), 6.63 (1H, br s), 6.64
(1H, s), 7.01 (1H, d, J = 6.8 Hz), 10.27 (1H, s)

¹³C NMR : δ ppm, 125 Mhz, in DMSO-d₆ : Figure 24, 25 (Page 81-82)
11.0, 22.5, 22.9, 28.8, 36.0, 36.5, 37.7, 41.3, 41.5, 48.5, 51.9, 55.3,
55.8, 61.0, 62.2, 101.6, 106.5, 109.2, 109.9, 110.9, 111.2, 126.6,
127.8, 129.9, 130.3, 138.6, 147.1, 146.8, 150.0

5.4 Characterization of AL-4

AL-4 was obtained as amorphous powder. It was soluble in chloroform and methanol.

EIMS : m/z (% relative intensity) : Figure 28 (Page 88)
450 (15.4), 259 (55.7), 230 (100), 191 (43.4)

IR : ν cm⁻¹ : KBr disc : Figure 29 (Page 89)
3419, 3245, 3077, 2945, 1427, 1283

- UV** : λ_{\max} nm (log ϵ) in methanol : Figure 30 (Page 90)
205 (3.52), 362 (2.73)
- $^1\text{H NMR}$** : δ ppm, 300 MHz., in DMSO- d_6 : Figure 31, 32,33, 34 (Page 93-96)
0.93 (3H, t, $J=9.2$ Hz), 3.66 (3H, s), 3.85 (3H, s), 6.55 (1H, s),
6.59 (1H, s), 6.91 (1H, s), 7.41 (1H, s), 9.14 (1H, br s)

5.5 Characterization of AL-1L mixture

- EIMS** : m/z (% relative intensity) : Figure 36 (Page 99)
475 (100), 288 (39.9), 262 (47.5), 208 (24.7), 185 (30.6)
- IR** : ν cm^{-1} : KBr disc : Figure 35 (Page 98)
3434, 3385, 3353, 3331, 2945, 2926, 1118
- $^1\text{H NMR}$** : δ ppm, 500 MHz., in DMSO- d_6 : Figure 37, 38, 39, 40, 41
(Page 101-105)
0.98 (3H, t, $J=5.6$), 1.09 (1H, m), 1.22(1H, m), 1.45 (1H, br d,
 $J=7.6$), 1.69 (1H, m), 1.72 (1H, m), 1.83 (1H, m), 2.07(1H, q,
 $J=9.2$), 2.28 (1H, m), 2.38 (1H, m), 2.67 (1H, m), 2.74 (1H, m),
2.87 (1H, m), 2.91 (1H, m), 2.96 (1H, m), 3.00 (1H, m), 3.08 (1H,
dd, $J=9.2, 3.2$), 3.42 (1H, dq, $J=10.0, 2.4$), 3.24 (3H, s),
3.66 (3H, s), 4.25 (1H, t, $J=4.0$), 5.87 (1H, s), 6.97 (1H, td,
 $J=6.0, 0.8$), 7.03 (1H, td, $J=6.0, 0.8$), 7.27 (1H, td, $J=6.0, 0.8$),
7.38 (1H, td, $J=6.0, 0.8$),
- $^{13}\text{C NMR}$** : δ ppm, 125 MHz., in DMSO- d_6 : Figure 42, 43 (Page 106-107)
AL-1L (A)
11.5, 23.1, 24.1, 24.7, 38.0, 38.1, 38.5, 43.4, 44.8, 53.5, 53.6, 56.1,
61.0, 62.5, 64.1, 100.6, 108.5, 115.7, 118.5, 112.0, 119.7, 122.0,
128.7, 134.2, 135.5, 137.3, 137.7, 148.6, 152.2,
AL-1L (B)
11.5, 20.4, 23.0, 24.5, 37.1, 38.5, 42.0, 43.3, 44.8, 52.1, 53.4, 55.7,
61.0, 63.8, 100.0, 113.4, 115.8, 117.9, 120.7, 121.1, 125.7, 126.4,
130.9, 134.0, 135.5, 139.0, 148.4, 152.1, 164.7