CHAPTER III

EXPERIMENTAL

Instruments.

1. Infrared Spectrophotometers:
   Perkin-Elmer model FT-IR 1760 X.
2. Nuclear Magnetic Resonance Spectrometer:
   Avance DPX - 300 (300 MH_Z).
   Jeol JNM - A500 (500 MH_Z).
3. Gas Chromatography Mass Spectrometer/ Mass Spectrometer:
   Varian Saturn GC/MS/MS / VG Platform II.
4. Melting Point Apparatus:
   Buchi capillary melting point apparatus.

Chemicals.

Acetic anhydride	(Anala R)
Acetone	(Sigma)
Andrographolide	
Benzoic acid	(Merck)
Butyric anhydride	(Sigma)
Chloroform	(Sigma)
4-Dimethylamino pyridine	(Fluka Chemika)
Ethyl acetate	(Sigma)
Heptanoic acid	(Sigma)
Hexane	(Sigma)

Methanol	(Sigma)
Sodium hydroxide	(Merck)
Stearic acid	(Merck)
Thionyl Chloride	(Laboratory grade)

All solvents used were either B.P. or Laboratory - grade.

1. Source of Plant Material.

The whole plant of *Andrographis paniculata* Nees. were collected from the campus of the Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in April 1997. The plant was identified by comparing with herbarium specimens in the Royal Forest Department, Ministry of Agriculture and Co-operatives, Bangkok, Thailand.

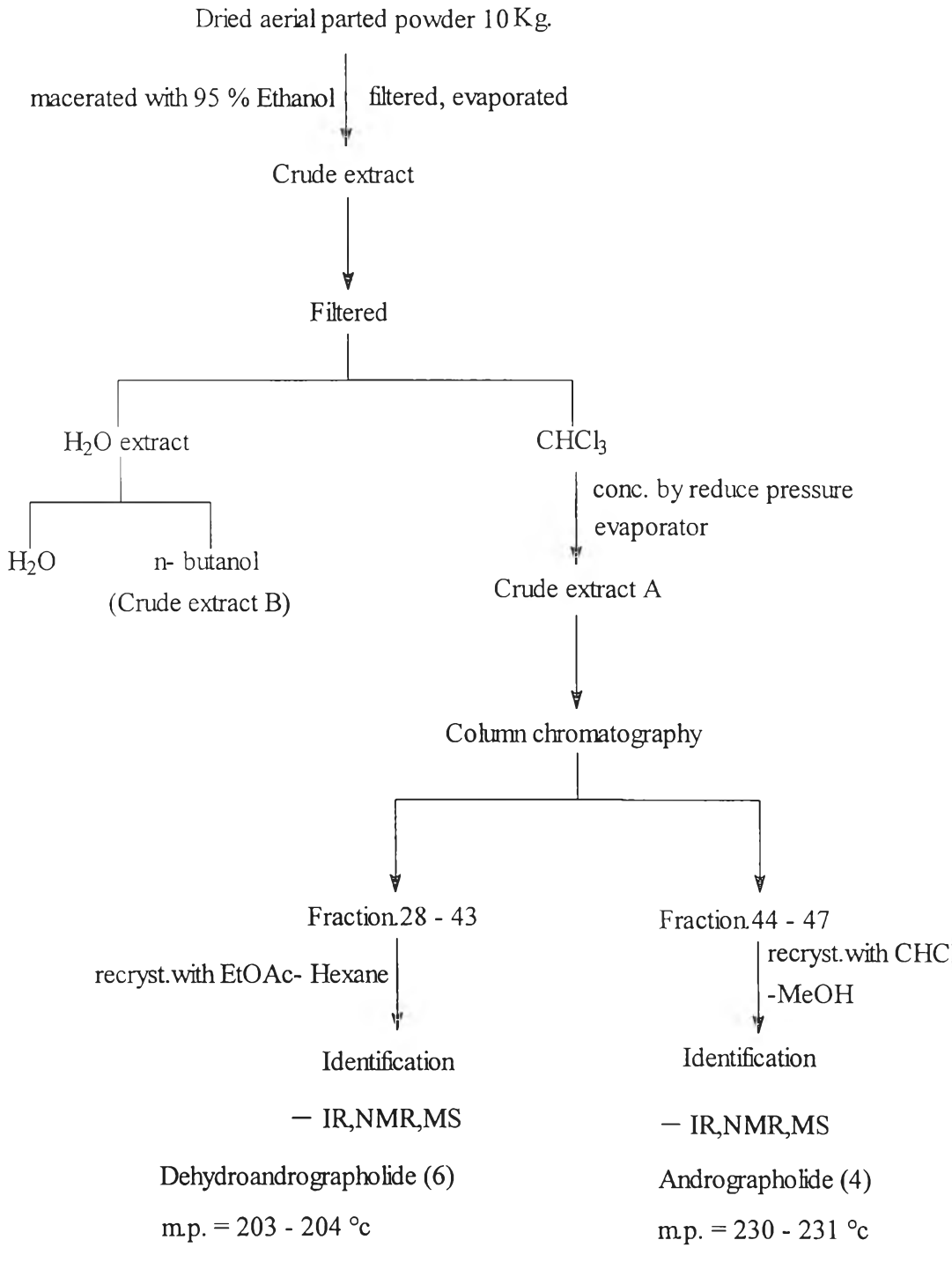
2. Extraction Procedure.

The dried aerial part powder of *Andrographis paniculata* Nees. (10 kg) were repeatedly macerated for four 3-day periods with 95% ethanol and then filtered. The filtrate from each maceration was evaporated under reduced pressure at temperature not exceeding 45 °C to yield 400 g of crude extract. The extract was dissolved in chloroform and partitioned with distilled water.

The chloroform extract was concentrated under reduced pressure evaporator to give a crude extract A (134g).

The water extract was partitioned with butanol to give butanol extract. The butanol extract was concentrated under reduced pressure evaporator to give a crude extract B (25 g).

The scheme of separation of *Andrographis paniculata* Nees. was performed as the following:



Scheme 3. Separation of *Andrographis paniculata* Nees.

3. Isolation Procedure

3.1 Isolation of chemical substance

The chloroform extract (134 g) was dissolved in a small amount of chloroform and methanol mixture and was loaded on a silica gel 60 column (2x52cm). The eluents were used in the order as shown below :

Chloroform : Hexane	1:1	1000 ml	fraction 1-27
Chloroform		1000 ml	fraction 28-34
Chloroform : Methanol	95:5	300 ml	fraction 35-43
		200 ml	fraction 44-47

Table 2. The combined fraction from crude chloroform extract.

Fraction number	Combined fraction	Weight (g)
1-27	C-1	15.32
28-43	C-2	24.00
44-47	C-3	35.50

3.2 Isolation of compound 4

Fraction 44-47 (35.50 g) was dissolved in a small amount of chloroform and methanol mixture and was loaded on a silica gel 60 column (2×52 cm). The column was eluted with mixture of chloroform : methanol (95 : 5). Thirty ml fraction were collected and combined after examination with TLC, using $CHCl_3$: MeOH (9:1) as TLC medium. Fraction 44-47 was separated from the combined fraction 1-30, was recrystallized in $CHCl_3$: MeOH (9:1) and MeOH. Compound 4 was collected at 230-231 °C and was obtained as colorless plates. The yield was 35.50 g (0.35% based on the dried weight of *Andrographis paniculata.*). This compound was identified as Andrographolide (4).

3.3 Isolation of compound 6

Fraction 28-43 was chromatographed over column chromatography (2.5× 30 cm) with silica gel 60 and eluted with $CHCl_3$: MeOH (95:5). Fraction with similar chromatographic pattern were combined as shown in table 2. Fraction C-2 showed one spot on TLC. It was recrystallized in EtOAc-Hexane to give 24.0 g of compound 6 (0.24% based on the dried weight of *Andrographis paniculata*), m.p. 203-204 °C as colorless needles. This compound was identified as 14-deoxy-11,12-didehydroan drographolide (6).

4. Esterification of Andrographolide (4)

Method

Method I.

Andrographolide (4) 1.0 g (0.0028 mole) was dissolved in 4 ml of redistilled pyridine in 50-ml round-bottom flask. Acetic anhydride was slowly added dropwise with vigorously stirring until the clear solution became opaque. The flask was fitted with refluxed condenser that connected to anhydrous calcium chloride guard tube at the top. The mixture was refluxed on a water bath for 2 or 3 hours. The mixture was evaporated by rotary evaporator. The residue was then extracted with 50 ml redistilled chloroform. The chloroform layer was separated with three 50 ml portions of purified water. The chloroform layer was separated and was dried with anhydrous sodium sulfate. The solvent was evaporated on a water bath (rotary evaporator), and then the residue was purified by column chromatography and preparative TLC.

Method II.

Andrographolide (4) 1.0 g (0.0028 mole) was dissolved in 10 ml of dichloromethane. Acid was added, together with 4-dimethylaminopyridine 0.1g(0.0008mole) as a catalyst. The flask was stopped with anhydrous calcium chloride tube. The entire apparatus was protected from the light, and the reaction mixture was stirred at room temperature (30°C) for at least 2 hours. After that, the reaction mixture was washed with sodium hydroxide (2x5 ml, 1mol/L), hydrochloric acid (2x5 ml, 1 mol/L) and then deionized water until an aqueous layer became neutral. The organic layer was dried with anhydrous sodium sulfate, evaporated and purified by column chromatography and preparative TLC.

4.1 Acetylation of Andrographolide (4)

Method I. Acetic anhydride 1.0 g (0.0097 mole) was added, and the reaction mixture was refluxed on a water bath for 2 hours. The product was extracted and purified using column chromatography and preparative TLC with mixture of chloroform : acetone (95 : 5)as eluent to obtain, 14-deoxy-11,12-didehydroandrographolide diacetate (A1) as yellow solid (0.22 g , 18.6 %), m.p. 134-135 °c.

Method II. Acetic anhydride 1.0 g (0.0097 mole) was added and the reaction was proceeded as above to obtain, 14-deoxy-11,12-didehydroandrographolide diacetate (A2) as yellow solid (0.32 g , 27.4%).

4.2 Acylation of Andrographolide (4)

4.2.1 Acylation of Andrographolide (4) with Butyric anhydride

Method I. Butyric anhydride (1.4 g , 0.009 mole) was added and the reaction mixture was refluxed for 3 hours. The product was extracted and purified by column chromatography and preparative TLC using the mixture of chloroform :

acetone (35 : 1). Analytical TLC of the crude product indicated two products; the less polar 14-deoxy-11,12-didehydroandrographolide dibutyrate (A3) was obtained as yellow waxy solid (0.16 g , 15 %) and the more polar component 14-deoxy-11,12-didehydroandrographolide monobutyrate (A4) was obtained as yellow waxy solid (0.17 g , 17.1 %).

Method II. Butyric anhydride (1.4 g, 0.009 mole) was added, and the reaction was proceeded as above to obtain only 14-deoxy-11,12-didehydroandrographolide dibutyrate (A5) as yellow waxy solid (0.46 g , 38.0 %).

4.2.2 Acylation of Andrographolide (4) with Benzoyl chloride

4.2.2.1 Preparation of Benzoyl chloride

Benzoic acid (25 g , 0.2 mole) and redistilled thionyl chloride (45g , 0.35 mole) was placed in a 250 ml round-bottom flask equipped with a refluxed condenser with a gas absorption trap at the top. The mixture was stirred and heated on a water bath with stirring until the evolution of sulfur dioxide and hydrogen chloride were ceased (about 5 hours). The mixture was distilled on oil bath to remove excess thionyl chloride (b.p. 67°C) and the benzoyl chloride was collected at 195-195 °C as colorless liquid (18 g , 65%).

4.2.2.2 Acylation of Andrographolide (4)

Method I. Benzoyl chloride (1.0 g , 0.007 mole) was added and the reaction mixture was refluxed for 5 hours. The product was extracted and purified by column chromatography and preparative TLC with mixture of chloroform : acetone (35:1) as eluent. Analytical TLC of the crude product indicated two products. The less polar 14-deoxy-11,12-didehydroandrographolide dibenzoate (A6) was obtained as yellow solid, m.p.174-175°C(0.14 g, 9%). The more polar component, 14-deoxy-

11,12-didehydroandrographolide monobenzoate (A7) was obtained as orange solid m.p. 160-161°C (0.36 g , 33.6 %).

Method II. Benzoyl chloride (1.0 g , 0.007 mole) was added and the reaction was proceeded as above. accepted that only the reaction mixture was stirred for 71 hours. 14-deoxy-11,12-didehydroandrographolide dibenzoate (A8) was obtained as yellow solid (0.52 g , 33.72%).

4.2.3 Acylation of Andrographolide (4) with Heptanoyl chloride

4.2.3.1 Preparation of Heptanoyl chloride

Heptanoic acid (20 g , 0.15 mole) and redistillated thionyl chloride (20 g , 0.15 mole) was placed in a 250 ml round-bottom flask equipped with a refluxed condenser with a gas absorption trap connect at the top. The mixture was stirred and heated on a water bath with stirring until the evolution of sulfur dioxide and hydrogen chloride were ceasd (about 2 hours). The mixture was distilled on oil bath to remove excess thionyl chloride (b.p. 67°C) and the heptanoyl chloride was collected at 173°C as colorless liquid (15 g , 65%).

4.2.3.2 Acylation of Andrographolide (4)

Method I. Heptanoyl chloride (1.3 g , 0.008 mole) was used. The reaction mixture was refluxed for 3 hours. extracted and purified by column chromatography and preparative TLC using mixture of chloroform : acetone (95:5) as eluent and the product, 14-deoxy-11,12-didehydroandrographolide diheptanoate (A9) was obtained as yellow waxy solid (0.14 g , 9.1%).

Method II. Heptanoyl chloride (1.3 g , 0.008 mole) was added and the reaction was proceeded as above to obtain, 14-deoxy-11,12-didehydroandro grapholide diheptanoate (A 10) as yellow waxy solid (0.42 g , 26.40%).

4.2.4 Acylation of Andrographolide (4) with Stearoyl chloride

4.2.4.1 Preparation of Stearoyl chloride

Stearic acid (20 g , 0.070 mole) and redistilled thionyl chloride (12 g , 0.073 mole) was placed in a 250 ml round-bottom flask equipped with a refluxed condenser and gas absorption trap connect at the top. The mixture was stirred heated on a water bath with stirring until the evolution of sulfur dioxide and hydrogen chloride were ceased (about 2 hours). The mixture was distilled on oil bath to remove excess thionyl chloride (b.p. 67 °C) and the stearoyl chloride was collected at 177-178 °C as colorless liquid (16 g , 75.13 %).

4.2.4.2 Acylation of Andrographolide (4)

Method I. Stearoyl chloride (2.6 g , 0.008 mole) was added. The reaction mixture was refluxed for 3 hours, extracted and purified using column chromatography and preparative TLC using mixture of $CHCl_3$: EtOAc (32:1) as eluent and 14-deoxy-11,12-didehydroandrographolide distearoate (A 11) was obtained as yellow waxy solid (0.24 g , 9.6%).

Method II. Stearoyl chloride (2.6 g , 0.008 mole) was added. The reaction was proceeded as above to obtain, 14-deoxy-11,12-didehydroandrographolide distearoate (A12) as yellow waxy solid (0.41 g , 16.7%).