



CHAPTER III

EXPERIMENTAL

1. SOURCE OF PLANT MATERIAL

The whole plant of Eupatorium adenophorum Spreng. (Compositae) was collected in May 1987 from Doi Suthep, Chiang Mai province, Thailand. Authentication was achieved by comparison with herbarium specimens at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand. A voucher specimen of plant material has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. GENERAL TECHNIQUES

2.1 Thin Layer Chromatography

Technique	:	one way , ascending
Absorbent	:	aluminium sheet silica gel 60 F ₂₅₄ pre-coated (E.Merck)
Plate size	:	5 x 8 cm
Layer thickness	:	0.2 mm
Solvent system	:	a) acetone : benzene (1:9) b) ethyl acetate : petroleum ether (3:7) c) ethyl acetate : benzene (1:9)

d) chloroform

e) chloroform : acetone (9:1)

Distance : 6 cm

Laboratory temperature : 24-30 °C

Detection on Chromatographic Plate :

1) Ultraviolet light at 254 nm.

The compounds which contain unsaturated bonds become visible as quenching spots under UV light at 254 nm.

2) Developing reagents

a) anisaldehyde spraying reagent (0.5 % methanolic solution of anisaldehyde with 5 % sulfuric acid)

The spots of sesquiterpenoids give specific colors with this reagent after heating at 110 °C for 2-4 min.

b) 10 % sulfuric acid in methanol

The spots of organic compounds give dark spot with this reagent after heating at 110 °C for 2-4 min.

2.2 Column Chromatography

Column size : 2.5 x 15 cm , 4.0 x 15 cm

Adsorbent : silica gel 0.040-0.063 mm (E. Merck)

- Packing of column : dry packing, then soaking with eluent
- Sample loading : the portion of crude extract was dissolved in small amount of organic solvent, added slowly on the top of a column.
- Solvent : a) 2-15 % acetone in benzene
b) 5-25 % ethyl acetate in petroleum ether
c) 0-10 % benzene in chloroform
d) methylene dichloride

2.3 Physical Constants

2.3.1 Melting Point

Melting point of EA-1 was determined on a Gallenkamp Melting Point Apparatus with digital thermometer Model-MFB-595 (uncorrected).

Melting point of EA-2 was determined on a Kofler-type hot stage apparatus (uncorrected).

2.3.2 Optical Rotation

Optical rotations were measured with a Perkin-Elmer 241 polarimeter.

2.4 Spectroscopy

2.4.1 Ultraviolet Spectra

Ultraviolet spectrum of EA-2 was recorded with a Beckman DU-7 spectrophotometer.

2.4.2 Infrared Spectra

Infrared spectrum of EA-1 was obtained on a Shimadzu Model IR 440 Spectrophotometer.

Infrared spectrum of EA-2 was obtained on a Nicolet' MX-1 interferometer.

2.4.3 Mass Spectra

Mass spectrum of EA-1 was recorded on a Mass Spectrometer Model DX 300 (Jeol) operating at 10 eV.

Mass spectrum of EA-2 was determined on a Varian MAT 112S double focusing mass spectrometer at 80 eV.

2.4.4 Nuclear Magnetic Resonance Spectra

For EA-1, proton (^1H) NMR spectrum was taken on a Jeol GX-270 (at 270 MHz) spectrometer with tetramethylsilane(TMS) as internal standard.

For EA-2, the ^1H -NMR spectra were obtained with either a Nicolet NMC 360 instrument operating at 360 MHz or Varian XL-300 instrument operating at 300 MHz.

The ^{13}C -NMR measurements of EA-2 were recorded with the Nicolet 360 instrument operating at 90.8 MHz.

Tetramethylsilane (TMS) was used as the internal standard and chemical shifts are reported as δ value (ppm). Homonuclear COSY spectra and heteronuclear HETCOR spectra were recorded on Varian XL-300 spectrometer. Standard Varian pulse sequences were used. The selective INEPT experiment were performed on the Nicolet NMC 360 spectrometer. Data sets of 16K covering spectra width of 10,000 Hz were acquired. Proton pulse width were calibrated by using a sample of active acid in 10% C_6D_6 ($^1r_J = 6.7$ Hz) in a 5 mm NMR tube. The ratio frequency field strength for the soft proton pulse was on the order of 25 Hz in these experiments. For 1-H and 2-H proton, 6 Hz was used as the 3J value and 4 Hz was used for the irradiation of the allylic and the

isopropyl group protons. Five thousand acquisitions were accumulated in each irradiation.

For EA-3, ^1H -NMR spectrum was obtained on Varian XL-300 instrument operating at 300 MHz.

3. Isolation of Chemical Substances from Eupatorium adenophorum Spreng. (Whole plants).

3.1 Extraction

The powdered dried plants of Eupatorium adenophorum Spreng. (2.1 kg) was extracted thoroughly by percolation with 95 % ethanol (20 liters). The percolate was evaporated under reduced pressure to yield a residue (90.3 g). The residue was treated with water (5 liter), followed by extraction with petroleum ether (3 times, 2 liter). The combined extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure to give a crude extract (40 g).

3.2 Isolation of Chemical Substances

The crude extract (40 g) was divided into 1.5-g portions. Each portion was chromatographed over a silica gel column (4 x 15 cm) using 0-15% acetone in benzene from as eluent. Fractions of 25 ml were

collected and examined by thin layer chromatography (TLC). Those fractions of similar pattern were combined and yielded four major fractions as follows :-

- a) Fractions 1-15 afforded a cruded mixture (1.10 g).
- b) Fractions 16-24 afforded a cruded mixture (480 mg).
- c) Fractions 25-42 afforded a cruded mixture (250 mg).
- d) Fractions 43-50 afforded a cruded mixture (620 mg).

The fractions 16-24 gave interested spot on TLC after treated with anisaldehyde spraying solution. This cruded mixture was further chromatographed over a silica gel column (2.5 x 15 cm) using benzene : chloroform (5 : 95) as eluent to yield EA-1 (70 mg) and EA-2 (90 mg).

The fractions 1-15 gave interested spot on TLC after treated with 10 % sulfuric acid in methanol spraying reagent. The crude mixture was further chromatographed over a silica gel column (2.5 x 15 cm) using ethyl acetate : petroleum ether (5 : 95) as eluent to yeild EA-3 (550 mg).

4. Characterization of the Isolated Compounds

The isolated compounds were characterized by the

data of hRf values, melting points, optical rotations, ultra-violet absorption spectra, infrared absorption spectra, mass spectra, nuclear magnetic resonance spectra and compared with previously published data of chemical compound.

4.1 Characterization of EA-1

EA-1 was obtained as a white amorphous powder. It is soluble in petroleum ether, ether, acetone and chloroform.

hRf Value

The hRf values given are obtained from the following systems:-

- a) acetone : benzene (1:9) = 56
- b) ethyl acetate : petroleum ether (3:7) = 65
- c) ethyl acetate : benzene (1:9) = 49
- d) chloroform = 65
- e) chloroform : acetone (9:1) = 68

(Fig. 3.1-3.5, p. 128-132)

Color Reaction

EA-1 gave purple color with anisaldehyde spraying solution on TLC aluminium sheet silica gel 60 F₂₅₄ pre-coated plate.

Melting Point

134-135 °C

Molecular Weight

414 (EIMS)

Infrared Absorbtion Spectrum (KBr disc)

ν_{\max} (cm^{-1})
 3520 (broad), 2950, 2850, 1650, 1450, 1390,
 1380, 1060, 1020 and 800 cm^{-1}

(Fig. 3.6, p. 133)

NMR Spectrum

The ^1H -NMR spectrum was performed in deuteriochloroform at 400 MHz in value (ppm).

(Fig. 3.7, p.134)

Mass Spectrum (EIMS)

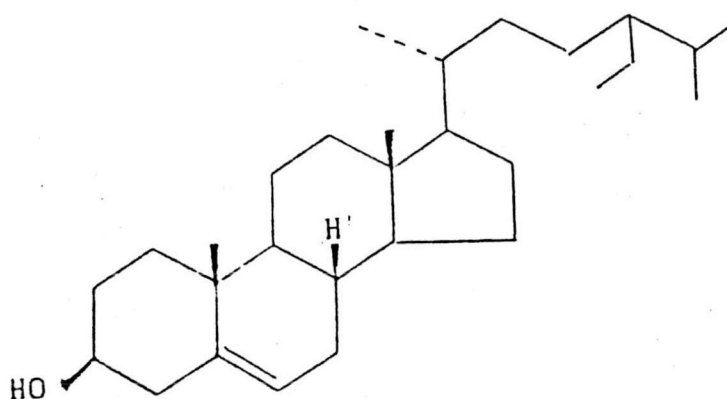
m/z (% relative intensity)
 414 (M^+ , 85), 399 (21), 396 (100), 381 (36), 329
 (29), 273 (25), 255 (61), 231 (19), 213 (41), 173 (21), 163
 (37), 161 (47), 159 (58), 147 (85), 135 (41), 133 (55), 131
 (43), 121 (53), 199 (51), 109 (48), 107 (75), 105 (79), 95
 (75), 93 (64), 91 (65), 83 (50), 81 (100), 43 (92).

(Fig. 3.8, p.135)

These data were identical with those obtained

for a sample of β -sitosterol, which had been isolated from Typha elephantina (141).

Therefore it is concluded that EA-1 is β -sitosterol, the structure of which is shown below.



4.2 Characterization of EA-2

EA-2 was crystallized from chloroform as white feather-like crystal. It is soluble in benzene, chloroform and ethyl acetate.

hRf value

The hRf given values are obtained from the following systems :-

a) acetone : benzene (1:9) = 51

b) ethyl acetate : petroleum ether (3:7) = 52

c) ethyl acetate : benzene (1:9) = 58

d) chloroform = 48

e) chloroform : acetone (9:1) = 44

(Fig. 3.1-3.5, p.128-132)

Melting Point

66-67 °C

Optical Rotation (MeOH, c = 1.3)

$[\alpha]_D +72.2$

Molecular Weight(EIMS)

234

Ultraviolet Absorption Spectrum (in MeOH)

λ_{\max} 242 nm (log 3.86)

(Fig. 3.9, p. 136)

Circular dichroism

The CD values in methanol were $[\theta]+2150$ and -840 at 248 and 300 nm, respectively.

(Fig. 3.10, p. 137)

Infrared Absorption Spectrum (KBr disc)

ν_{\max} (cm⁻¹)

1725, 1710, 1605

(Fig. 3.11, p. 138)

NMR Spectrum

see Table 5.

(Fig. 3.12-3.19, p. 139-146)

Table 5. ^1H - and ^{13}C -NMR Assignments of EA-2

Carbon	^1H (ppm)	^{13}C (ppm)
1	8.35(d, 1.5)	146.89
2	3.22(m)	41.18
3	2.03(m)	63.90
4	-	212.87
5	2.54(dd, 16.6, 4.5)	41.04
5	2.81(dd, 16.6, 3.4)	
6	2.28(m)	39.24
7	2.05(m)	28.10
8	2.03(dd, 8.8, 4.2)	45.80
8	2.20(d, 8.8)	
9	-	198.10
10	-	136.00
11	2.00(m)	31.78
12	0.88*(d, 6.1)	19.97*
13	1.08*(d, 6.1)	20.28*
14	1.02(d, 6.4)	20.93
15	1.73(d, 1.4)	15.43

Spectra were recorded in CDCl_3 .

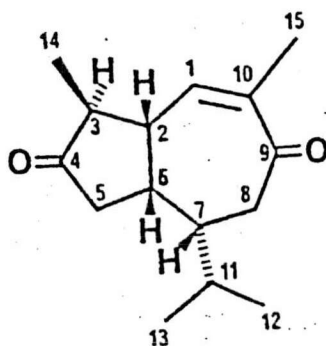
* Assignment may be interchanged.

Mass Spectrum (EIMS)

m/z (% relative intensity)
 234 (M^+ , 22), 232 (6), 216 (10), 193 (41), 177
 (6), 164 (9), 150 (45), 136 (84), 121 (28), 109 (24), 89
 (100), 55 (26)

(Fig. 3.20, p. 147)

The results were concluded for the structure of EA-2 as a new sesquiterpene skeleton and the chemical structure was assigned as $C_{15}H_{22}O_2$ and named eupatorenone. The structure of which is shown below.



4.3 Characterization of EA-3

EA-3 was obtained as semisolid substance. It was soluble in ether, benzene, petroleum ether and acetone.

hRf Value

The hRf values given are obtained from the

following systems :-

- a) acetone : benzene (1:9) = 65
 - b) ethyl acetate : petroleum ether (3:7) = 79
 - c) ethyl acetate : benzene (1:9) = 72
 - d) chloroform = 76
 - e) chloroform : acetone (9:1) = 90
- (Fig. 3.1-3.5, p.128-132)

Color Reaction

EA-3 gave orange color with anisaldehyde spraying reagent on TLC aluminium sheet silica gel 60F₂₅₄ precoated plate.

NMR Spectrum

EA-3 gave NMR spectrum shown in Fig. 3.21, p. 148.

The results were concluded for the structure of EA-3 as a triterpenoid. The further elucidation is not performed.