



CHAPTER III

EXPERIMENTAL

1. SOURCE OF PLANT MATERIAL

The fruits and stems of *Piper ribesioides* Wall. were collected from Chumphorn Province, Thailand, in July, 1988 and authenticated by comparison with herbarium specimens at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, 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 (TLC)

Technique : one way, ascending

Absorbent : aluminium sheet silica gel 60 F₂₅₄
pre-coated (E.Merck)

Plate size : 5 x 5 cm

Layer thickness : 0.2 mm

Solvent system : a) hexane : acetone (9:1)
b) hexane : chloroform (7:3)
c) hexane : chloroform (3:7)
d) benzene
e) chloroform
f) chloroform : methanol (95:5)

Distance : 4 cm

Laboratory Temperature : 24 - 30°C

Detection on chromatographic plate :

a) Ultraviolet light at 254 nm

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

b) Developing reagents

1) Anisaldehyde spraying reagent

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

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

2) 10% Sulfuric acid in methanol

The spots of organic compounds give dark spot with this reagent after heating at 100°C for 2-4 min.

2.2 COLUMN CHROMATOGRAPHY

Column size : 2.5 cm x 15 cm, 4 cm x 15 cm

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

Packing of column : dry packing

Sample loading : The portion of crude extract was dissolved in small amount of organic solvent, mixed with small quantity of adsorbent, dried,

trituated and added onto the top
of a column.

Solvents : a) chloroform
 b) benzene : chloroform (7:3)
 c) benzene : ethanol (98:2)

2.3 PHYSICAL CONSTANT

Melting point : melting points of the isolated compounds were determined by Gallenkamp Melting Point Apparatus.

2.4 SPECTROSCOPY

2.4.1 Infrared (IR) absorption spectra

The infrared absorption spectra were obtained on a Nicolet Model 20 SX/C FTIR spectrometer.

2.4.2 Nuclear magnetic resonance (NMR) spectra

The proton (^1H) and carbon-13 (^{13}C) NMR spectra were taken on a Bruker WH-400 Spectrometer with standards respectively. The multiplicities for ^{13}C NMR spectra were determined by the attached proton test which produced positive (+) quaternary C and CH_2 signals and negative (-) CH and CH_3 signals.

2.4.3 Mass spectra

The mass spectra were obtained on a VG Micromass 7070 F or a ZAB-E Spectrometer.

3. EXTRACTION AND PURIFICATION

3.1 FRUITS OF *PIPER RIBESIOIDES* WALL.

The dry fruits of *Piper ribesoides* Wall. (200 g) were blended with ethanol, macerated twice for 7 day-periods (1.5L and 1L) and filtered by suction. The combined filtrate was evaporated under reduced pressure to dryness. The residue was suspended in water (500ml) and extracted with chloroform (5x200ml). The combined chloroform fractions, after drying (Na₂SO₄ anhydrous) and evaporation, yielded 30 gm of syrupy mass (crude FP).

The crude FP was divided into 12 equal portions and each one was treated in the following. Each portion was chromatographed on a silica gel column (4x15cm) using chloroform as eluent. Twenty five millilitre of each fraction was collected and compared by TLC. Those fractions of similar pattern were combined and evaporated to dryness. Fractions 15-46 and 47-78 after evaporation, afforded residue FPa and FPb respectively.

3.1.1 Residue FPa was rechromatographed on silica gel (2.5x15cm) column using benzene : chloroform 7:3 as eluent to furnish 220mg (0.11%) of FP-1.

3.1.2 Residue FPb was rechromatographed on silica gel (2.5x15cm) column using benzene : ethanol 98:2 as eluent to furnish 500mg (0.25%) of FP-2.

3.2 STEMS OF *PIPER RIBESIOIDES* WALL.

The dry stems of *Piper ribesoides* Wall. (2kg) were blended with chloroform, macerated twice for 7 day-periods (4L and 4L) and filtered by suction. The combined filtrate was evaporated under

reduced pressure to dryness. The residue was suspended in water (500ml) and extracted with chloroform (5x200ml). The combined chloroform fractions, after drying (Na_2SO_4 anhydrous) and evaporation, yielded 30 gm of syrupy mass (crude SP).

The crude SP was divided into 12 equal portions and each was treated in the same manner. Each portion was chromatographed on a silica gel column (4x15cm) using chloroform as eluent. Twenty five millilitres of each fraction was collected and compared by TLC. Those fractions of similar pattern were combined and evaporated to dryness. Fractions 5-30 and 35-84 after evaporation, afforded residue SPa and SPb respectively.

3.2.1 Residue SPa was rechromatographed on silica gel (2.5 x 15 cm) column using chloroform as eluent.

3.2.1.1 Fractions 5-7 were designated as SP-1 (30 mg, 0.0015%)

3.2.1.2 Fractions 10-12 were designated as SP-2 (40 mg, 0.0020%)

3.2.1.3 Fractions 14-15 were designated as SP-3 (40 mg, 0.0020%)

3.2.2 Residue SPb was rechromatographed on silica gel (2.5 x 15 cm) column using benzene : chloroform 7:3 as eluent.

3.2.2.1 Fractions 15-21 were designated as SP-4 (30 mg, 0.0015%)

3.2.2.2 Fractions 26-39 were designated as SP-5 (56 mg, 0.0028%)

3.2.2.3 Fractions 43-46 were designated as SP-6 (36 mg, 0.0018%)

4. CHARACTERIZATION OF THE ISOLATED COMPOUNDS

The isolated compounds were characterized by the data of hRf values, melting points, infrared absorption spectra mass spectra nuclear magnetic resonance spectra and compared with previously published data of known chemical compounds.

4.1 CHARACTERIZATION OF FP-1

FP-1 was obtained as a viscous liquid. It is soluble in hexane, chloroform and methanol.

hRf value

The hRf values given are obtained from the following systems :

a) hexane : acetone	(9:1) = 8
b) hexane : chloroform	(7:3) = 8
c) hexane : chloroform	(3:7) = 38
d) benzene	= 18
e) chloroform	= 69
f) chloroform : methanol	(95:5) = 100

(Fig. 24-29, p. 205-210)

Molecular weight

354 (EIMS)

Infrared absorption spectrum (in CCl₄)

ν_{\max} (cm⁻¹)

3069, 3009, 2957, 2950, 2884, 2775, 1776, 1768, 1696,
1619, 1500, 1486, 1441, 1092, 1040.

(Fig. 30, p. 211)

Proton NMR Spectrum (in CDCl₃, 400 MHz)

Chemical Shifts (δ) ppm	Proton	Multiplicity
2.44	7 ₂ -CH ₂ (1H)	m
2.47	8-CH (1H)	m
2.54	8'-CH (1H)	m
2.60	7 ₁ -CH ₂ (1H)	m
2.84	7 ₂ ^I -CH ₂ (1H)	dd
2.99	7 ₁ ^V -CH ₂ (1H)	dd
3.86	9 ₂ -CH ₂ (1H)	dd
4.12	9 ₁ -CH ₂ (1H)	dd
5.94	-O-CH ₂ -O- (4H)	s
6.46	3,5-CH (2H)	m
6.60	5'-CH (1H)	dd
6.63	3'-CH (1H)	d
6.70	6-CH (1H)	d
6.75	6'-CH (1H)	d

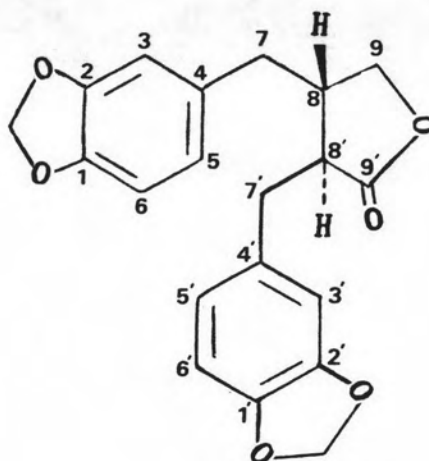
(Fig. 31, p. 212)

Mass spectrum (EIMS)

m/z (% relative intensity)

354 (M⁺, 25), 218 (12), 192 (15), 162 (14),
135 (100), 105 (7), 83 (96).

From the above data, FP-1 was in complete agreement with the structure of hinokinin (Belletire and Fry, 1987 ; Jakupovic, 1986). It is therefore conclude that FP-1 is hinokinin, the structure of which is shown below.



Hinokinin

4.2 CHARACTERIZATION OF FP-2

FP-2 was obtained as a white amorphous powder. It is soluble in acetone, chloroform and methanol.

hRf value

The hRf values given are obtained from the following systems :

a) hexane : acetone	(9:1) = 13
b) hexane : chloroform	(7:3) = 5
c) hexane : chloroform	(3:7) = 20
d) benzene	= 10
e) chloroform	= 30
f) chloroform : methanol	(95:5) = 79

(Fig. 24-29, p. 205-210)

Melting point

151°C [Lit. 153-154°C (Box and Chan, 1975)]

Molecular weight

300 (EIMS)

Microanalysis

found C = 75.99%, H = 8.13%, N = 0.04%

calculation for $C_{19}H_{24}O_3$ C = 75.97%, H = 8.03%**Infrared absorption spectrum (in $CHCl_3$)** ν_{max} (cm^{-1})3574, 3288, 2989, 2942, 2869, 1684, 1631, 1598, 1578,
1505, 1318, 1265, 1165, 1105, 972, 826.

(Fig. 32, p. 213)

Proton NMR Spectrum (in $CDCl_3$, 400 MHz)

Chemical Shifts (δ) ppm	Proton	Multiplicity	J (Hz)
0.88	CH ₃ (3H)	s	
0.90	CH ₃ (3H)	s	
0.94	CH ₃ (3H)	s	
1.05		dd	13.1, 3.4
1.32		m	
1.71	3-6-CH, CH ₂ (7H)	t	
1.78		m	
2.05		m	
2.42		m	

5.01	1-CH (1H)	dt	10.0, 2.8
5.50	7'-OH (1H)	s	
6.33	2'-CH (1H)	d	16
6.85	5',9'-CH (2H)	dd	8.6, 1.9
7.45	6'-8'-CH (2H)	dd	8.6, 1.9
7.62	3'-CH (1H)	d	16

(Fig. 33. p. 214)

Mass spectrum (EIMS)

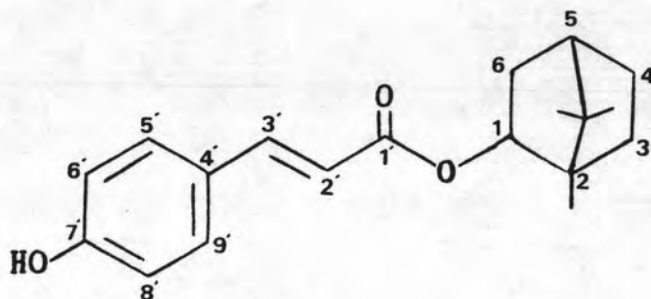
m/z (% relative intensity)

300 (M^+ , 14), 164 (5), 147 (100), 119 (12),

83 (32), 69 (7), 47 (7).

(Fig. 34, p. 215)

From the above data, FP-2 was in complete agreement with the structure of bornyl *p*-coumarate (Hernandez *et al.*, 1986) [or the other name is borneol *trans-p*-hydroxycinnamate (Dukhovlina, Sklyar and Sdobnina, 1975)]. It is therefore concluded that FP-2 is Bornyl *p*-coumarate, the structure of which is shown below.



Bornyl *p*-coumarate

4.3 CHARACTERIZATION OF SP-1

SP-1 was obtained as a yellow amorphous powder. It is soluble in benzene, chloroform and methanol.

hRf value

The hRf values given are obtained from the following systems :

a) hexane : acetone	(9:1) = 43
b) hexane : chloroform	(7:3) = 33
c) hexane : chloroform	(3:7) = 83
d) benzene	= 53
e) chloroform	= 88
f) chloroform : methanol	(95:5) = 100

(Fig. 24-29, p. 205-210)

Melting point

108 - 109.5°C [Lit. 104-105°C (Kijjoa, *et al.*, 1989)]

Molecular weight

214 (EIMS)

Infrared absorption spectrum (in CHCl₃)

ν_{\max} (cm⁻¹)

3025, 2945, 1703, 1629, 1609, 1263, 1218, 1141

Proton NMR spectrum (in CDCl₃, 400 MHz)

Chemical Shifts (δ) ppm	Proton	Multiplicity	J (Hz)
3.76	-OCH ₃ (3H)	s	
5.92	2-CH (1H)	d	15.2
6.44	4-CH (1H)	dd	15.1, 11.3
6.72	5,7-CH (2H)	d&dd	
6.87	6-CH (1H)	dd	15.4, 10.7
7.27	4'-CH (1H)	m	
7.38	3',5'-CH (2H)	br, t	
7.39	3-CH (1H)	dd	15.3, 11.4
7.43	2',6' CH (2H)	dd	

(Fig. 35, p. 216)

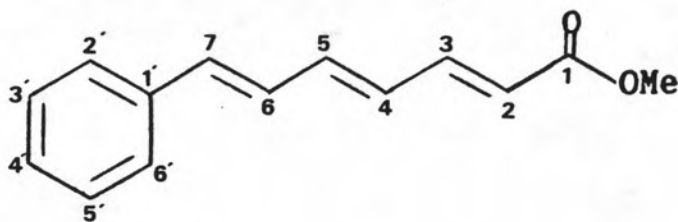
Mass spectrum (EIMS)

m/z (% relative intensity)

214 (M⁺, 54), 181 (14), 165 (3), 155 (100),
 141 (3), 128 (14), 115 (11), 107 (3), 91 (9),
 77 (11), 44 (4).

(Fig. 36, p. 217)

From the above data, SP-1 was in complete agreement with the structure of methyl 2E,4E,6E-7-phenyl-2,4,6-heptatrienoate (Asokan, Ila and Junjappa, 1985 ; Kijjoa *et al.*, 1989). It is therefore concluded that SP-1 is methyl 2E,4E,6E -7-phenyl-2,4,6-hepta-trienoate, the structure of which is shown below.



Methyl 2E,4E,6E -7-phenyl-2,4,6-heptatrienoate

4.4 CHARACTERIZATION OF SP-2

SP-2 was obtained as a yellow amorphous powder. It is soluble in benzene, chloroform and methanol.

hRf value

The hRf values given are obtained from the following systems :

- | | |
|--------------------------|--------------|
| a) hexane : acetone | (9:1) = 30 |
| b) hexane : chloroform | (7:3) = 23 |
| c) hexane : chloroform | (3:7) = 75 |
| d) benzene | = 43 |
| e) chloroform | = 83 |
| f) chloroform : methanol | (95:5) = 100 |

(Fig. 24-29, p. 205-210)

Melting point

142 - 143°C [Lit. 145-146°C (Gupta, *et al.*, 1972b)]

Molecular weight

232 (EIMS)

Infrared absorption spectrum (in CHCl_3) ν_{max} (cm^{-1})

3025, 3002, 2925, 2857, 1738, 1468, 1168, 1098.

(Fig. 37, p. 218)

Proton NMR Spectrum (in CDCl_3 , 400 MHz)

Chemical Shifts (δ) ppm	Proton	Multiplicity	J (Hz)
3.76	-OCH ₃ (3H)	s	
5.95	2-CH (1H)	d	15.3
5.99	-O-CH ₂ -O- (2H)	s	
6.70	4-CH (1H)	dd	15.2, 10.9
6.79	5'-CH (1H)	d	7.6
6.81	5-CH (1H)	d	15.4
6.91	6'-CH (1H)	dd	1.4
7.00	2'-CH (1H)	d	1.4
7.42	3-CH (1H)	dd	15.2, 10.7

(Fig. 38, p. 219)

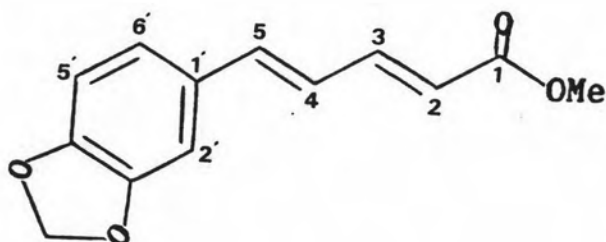
Mass spectrum (EIMS)

 m/z (% relative intensity)

232 (M^+ , 18), 214 (53), 173 (22), 155 (100),
 129 (11), 115 (19), 91 (18), 71 (11), 57
 (29), 43 (14).

(Fig. 39, p. 220)

From The above data, SP-2 was in complete agreement with the structure of methyl piperate (Kijjoa *et al.*, 1989). It is therefore concluded that SP-2 is methyl piperate, the structure of which is shown below.



Methyl piperate

4.5 CHARACTERIZATION OF SP-3

SP-3 was obtained as a white amorphous powder. It is soluble in benzene, chloroform and methanol.

hRf value

The hRf values given are obtained from the following systems :

- | | |
|--------------------------|--------------|
| a) hexane : acetone | (9:1) = 10 |
| b) hexane : chloroform | (7:3) = 10 |
| c) hexane : chloroform | (3:7) = 50 |
| d) benzene | = 30 |
| e) chloroform | = 73 |
| f) chloroform : methanol | (95:5) = 100 |

(Fig. 24-29, p. 205-210)

Molecular weight

324 (EIMS)

Infrared absorption spectrum (in CHCl₃)

SP-3 gave IR absorption spectrum shown in Fig. 40, p. 221

Proton NMR spectrum (in CDCl₃, 400 MHz)

SP-3 gave proton NMR spectrum shown in Fig. 41, p. 222

Mass spectrum (EIMS)

SP-3 gave mass spectrum shown in Fig. 42, p. 223

The structure elucidation of this compound is in progress.

4.6 CHARACTERIZATION OF SP-4

SP-4 was obtained as a white feather-like crystal. It is soluble in chloroform, ethanol and methanol.

hRf value

The hRf values given are obtained from the following systems :

a) hexane : acetone	(9:1) = 0
b) hexane : chloroform	(7:3) = 0
c) hexane : chloroform	(3:7) = 15
d) benzene	= 0
e) chloroform	= 26
f) chloroform : methanol	(95:5) = 89

(Fig. 24-29, p. 205-210)

Molecular weight

279 (EIMS)

Infrared absorption spectrum (in CHCl₃)

SP-4 gave IR absorption spectrum shown in Fig. 43, p. 224

Proton NMR Spectrum (in CDCl₃, 400 MHz)

SP-4 gave Proton NMR spectrum shown in Fig. 44, p. 225

Carbon-13 NMR spectrum (in CDCl₃, 400 MHz)

SP-4 gave C-13 NMR spectrum shown in Fig. 45, p. 226

Mass spectrum (EIMS)

SP-4 gave mass spectrum shown in Fig. 46, p. 227

The structure elucidation of this compound is in progress.

4.7 CHARACTERIZATION OF SP-5

SP-5 was obtained as a white needle-like crystal. It is soluble in chloroform, ethanol and methanol.

hRf value

The hRf values given are obtained from the following systems :

- | | |
|------------------------|------------|
| a) hexane : acetone | (9:1) = 0 |
| b) hexane : chloroform | (7:3) = 0 |
| c) hexane : chloroform | (3:7) = 10 |

d) benzene	= 0
e) chloroform	= 21
f) chloroform : methanol	(95:5) = 76

(Fig. 24-29, p. 205-210)

Molecular weight

414 (EIMS)

Infrared absorption spectrum (in CHCl₃)

SP-5 gave IR absorption spectrum shown in Fig. 47, p. 228

Proton NMR Spectrum (in CDCl₃, 400 MHz)

SP-5 gave Proton NMR spectrum shown in Fig. 48, p. 229

Mass spectrum (EIMS)

SP-5 gave mass spectrum shown in Fig. 49, p. 230

The results were concluded for the structure of SP-5 as a steroid. Further elucidation of the absolute structure is in progress.

4.8 CHARACTERIZATION OF SP-6

SP-6 was obtained as a yellow semisolid. It is soluble in chloroform, ethanol and methanol.

hRf value

The hRf values given are obtained from the following systems :

a) hexane : acetone	(9:1) = 0
b) hexane : chloroform	(7:3) = 0
c) hexane : chloroform	(3:7) = 5
d) benzene	= 0
e) chloroform	= 10
f) chloroform : methanol	(95:5) = 68

(Fig. 24-29, p. 205-210)

Molecular weight

357 (EIMS)

Infrared absorption spectrum (in CHCl₃)

SP-6 gave IR absorption spectrum shown in Fig. 50, p. 231

Proton NMR spectrum (in CDCl₃, 400 MHz)

SP-6 gave proton NMR spectrum shown in Fig. 51, p. 232

Mass spectrum (EIMS)

SP-6 gave mass spectrum shown in Fig. 52, p. 233

The structure elucidation of this compound is in progress.