CHAPTER IV

EXPERIMENTAL

Source of Plant Materials

The stems of *Strychnos thorelii* Pierre ex Dop, which used in this study, were collected from Soi Dao Waterfall, Khao Soi Dao Wildlife Sanctuary, Chantaburi Province, Thailand in January 1994. The plant material was authenticated by comparison with the specimens at Royal Forest Department, Bangkok, Thailand.

General Techniques

1. Thin-Layer Chromatography (TLC)

Technique : one way, ascending

Adsorbent : mixture of silica gel 60 G (E.Merck) 20 g and silica gel 60 HF_{254} (E.Merck) 10 g in 60 ml distilled water

Plate sizes : 5x20 cm , 10x20 cm , 20x20 cm

Layer thickness : 0.25 mm

Activation : air -dried for 15 minutes and then heated in hot air oven at 110 °c for 2 hours.

Solvent systems : 1. Chloroform : Methanol (8:2)

2. Methanol : Benzene (1:2)

3. Methanol

4. Acetone

5. Ethyl acetate

6. Petroleum ether : Acetone (7:3)

7. Chloroform : Acetone (9:1)

8. Hexane : Ethyl acetate (2:1)

9. Chloroform : Methanol (15:1)

10. Hexane : Acetone (1:1)

Distance : 15 cm

Laboratory temperature : 30-35 °c

Development :The plates were developed in chromatographic tank lined with filter paper.

Detection on chromatographic plate :

1. Visual detection under daylight.
2. Visual detection under ultraviolet light at wavelengths 254 and 366 nm
3. Chromogenic agents

3.1 Ferric chloride-perchloric acid spraying reagent

(1 ml of 0.5 M ferric chloride solution in 100 ml of 35% aqueous perchloric acid solution)

3.2 Dragendorff 's spraying reagent

(1 ml stock solution in 2 ml glacial acetic acid and 7 ml distilled water

The stock solution : mixture of bismuth oxynitrate 1.7 g, glacial acetic acid 20 ml, distilled water 80 ml and 5% aqueous potassium iodide 100 ml)

2. Quick Column Chromatography

Column size : scintered-glass filter column of diameter 15 cm

Adsorbent : silica gel 60 particle size 0.040 - 0.063 mm (E.Merck)

packing method : wet packing

Solvent system : chloroform : methanol : ammonium hydroxide (90:10:0.5)

3. Column Chromatography (CC)

Column size : The glass columns in various diameters depending on the quantity of sample.

Adsorbent : silica gel 60 particle size 0.040-0.063 mm (E.Merck)

Packing method : wet packing

Solvent systems : 1. Chloroform : Ethyl acetate : Ammonium hydroxide (90:10:0.2)

2. Hexane : Methanol : Chloroform (7:1:2)

3. Ethyl acetate : Hexane (1:2)

4. Chloroform : Methanol: Ammonium hydroxide (95:5:1)

5. Hexane : Ethyl acetate : Methanol (2:1:0.2)

4. Melting Point

Melting point of the isolated compounds were determined on a Buchi melting point apparatus.

5. Ultraviolet (UV) Absorption Spectroscopy

The spectra were determined on a Hitachi UV-220 A spectrophotometer.

6. Infrared (IR) Absorption Spectroscopy

The spectra were determined on a Perkin Elmer FT-IR 1760 X infrared spectrophotometer.

7. Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H NMR , ^{1}H-^{1}H COSY and ^{13}C NMR spectra were determined on a JEOL JNM-A 500 spectrometer.

8. Mass Spectroscopy (MS)

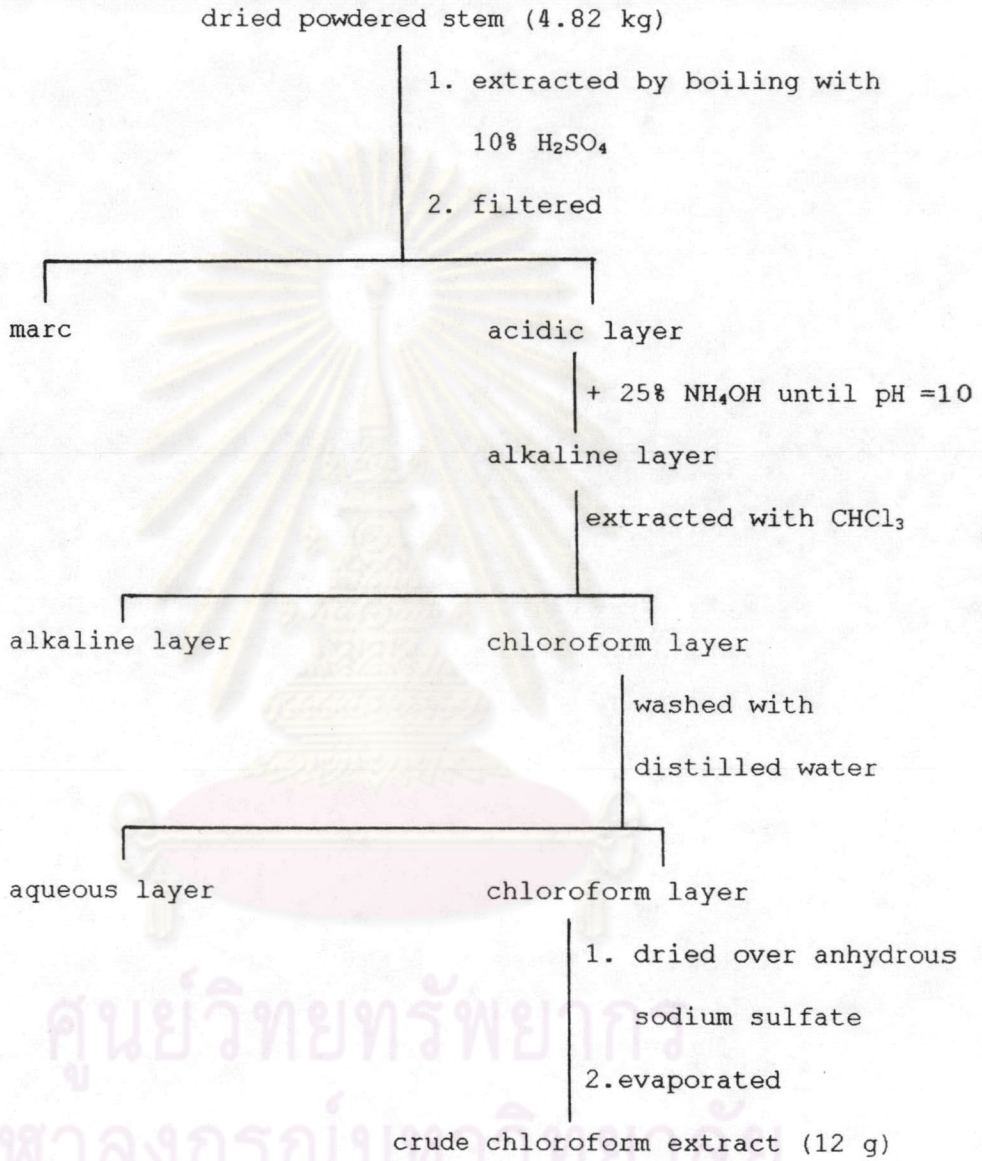
The mass spectra were determined on a JEOL-JMS mass spectrometer model DX 300.

9. Solvent

The solvents used in chromatographic techniques were redistilled before used.

Extraction (scheme 1)

The dried powdered stems of *Strychnos thorelii* Pierre ex Dop (4.82 kg) were divided into 8 portions. Each portions (600 g) was extracted by boiling with 10% sulfuric acid (3 liters) for 2 hours. The acidic filtrate was cooled and basified with 25% ammonium hydroxide solution to approximately pH 10. The alkaline solution was extracted with chloroform. The chloroform layer was washed with small amount of distilled water (20 ml). Then the chloroform layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to dryness to give a crude chloroform extract (12 g).



Scheme 1 Extraction Procedure of *Strychnos thorelii* Pierre ex Dop

Separation and Isolation of the Chemical Constituents

The crude chloroform extract (12g) was divided into 2 portions. Each portion was purified by quick column chromatographic technique using a scintered - glass filter column of silica gel G. The solvent mixture consisted of chloroform , methanol , and ammonium hydroxide (90:10:0.5) was used as eluents , ninety-five fractions (50 ml each) were collected. The column was washed with methanol until the eluates were exhausted and clear. Each fraction was examined by thin layer chromatography (TLC). Fractions which indicated similar pattern were combined together . The separation profile of the column was summarized as follows.

Portion		Eluent
A	(1-15)	$CHCl_3$:MeOH:NH_4OH (90:10:0.5)
B	(16-31)	"
C	(32-71)	"
D	(72-95)	"
E	(96-136)	MeOH

Portion A : This portion contained yellow compounds which gave orange spots with dragendorff's reagent , so it was further investigated .

Portion B : This portion contained mainly a compound which gave greyish-blue color with ferric chloride-perchloric acid reagent after heating at 110 °c for 30 minutes . It was further investigated.

Portion C : This portion contained the compound occurred in portion B which gave greyish-blue color with ferric chloride-perchloric acid reagent. Because of the small amount of this compound and some impurities included , this portion was not further investigated.

Portion D : This portion was not further investigated due to the small amount of substances obtained.

Portion E : This portion contained the large amount of dirty mass , so it was not further investigated.

Isolation of ST-1 from portion B

Portion B (1.46 g) was separated by the column chromatographic technique using a column of silica gel G (80 g). By eluting the column with chloroform : methanol : ammonium hydroxide (95:5:1) , seventy - five fractions (20 ml each) were collected. The separation profile was arranged as follows.

Portion	Eluent
BA (1-20)	$CHCl_3$:MeOH:NH_4OH (95:5:1)
BB (21-33)	"
BC (34-42)	"
BD (43-59)	"
BE (60-75)	MeOH

Portion BC gave greyish-blue spot on TLC after treated with ferric chloride - perchloric acid spraying reagent. While the other portions was not clean and contained lot of components.

Portion BC (410 mg) was chromatographed over silica gel G (50 g) and eluted with hexane : ethyl acetate : methanol (2:1:0.2). Fifty fractions (10 ml each) were collected. The successive elution was summarized as follows.

Portion	Eluent
BC_1 (1-9)	Hexane:EtOAc:MeOH (2:1:0.2)
BC_2 (10-14)	"
BC_3 (15-24)	"
BC_4 (25-40)	"
BC_5 (41-50)	MeOH

Crystallization of component of the portion BC_3 yielded a pure compound. It was recrystallized from methanol as white plate crystal. It yielded 206 mg (0.0042 %) and was designated as ST-1.

Isolation of ST-2 from portion A

Portion A (2.5g) was fractionated by the column chromatographic technique using a column of silica gel G (100 g). By eluting the column with chloroform : ethyl acetate : ammonium hydroxide (90:10:0.2) , thirty-five fractions (20 ml each) were collected. The separation profile was demonstrated as follows.

Portion	Eluent
AA (1-3)	$CHCl_3$:EtOAc:NH_4OH (90:10:0.2)
AB (4-10)	"
AC (11-15)	"
AD (16-28)	"
AE (29-35)	MeOH

Thin layer chromatographic patterns indicated that the two yellow compounds were found as major components in portion AB. While the other portions contained less quantity of the mentioned compounds with large amount of impurities , so only portion AB was further investigated.

Portion AB (1.36 g) was chromatographed over silica gel G (80 g) and eluted with hexane : methanol : chloform (7:1:2). Ten fractions (20 ml each) were collected and the separation profile was summarized as follows.

Portion	Eluent
AB_1 (1)	Hexane:MeOH:$CHCl_3$ (7:1:2)
AB_2 (2-4)	"
AB_3 (5-10)	"

A yellow compound was crystallized from portion AB_2. Unfortunately, it could not be isolated as pure compound . So the further purification was carried out .

Portion AB_2 (750 mg) was chromatographed over silica gel G (80 g) and eluted with ethyl acetate : hexane (1:2). Twenty - five fractions (10 ml each) were collected and the separation profile was summarized as follows.

Fraction	Eluent
1-10	EtOAc:Hexane (1:2)
11-16	"
17-25	"

A yellow compound was crystallized from fractions 11-16 . It was recrystallized from acetone as yellow needle crystals. It yielded 440 mg (0.0091 %) and was designated as ST-2.

Characterization and Identification of ST-1

ST-1 was obtained as white plate crystal. It is soluble in methanol. It gave greyish - blue color with ferric chloride - perchloric acid spraying reagent followed by heating at 110 °c for 30 minutes.

Melting point

189-190 °c (decomposed)

Molecular weight

420

Empirical formula

$C_{22}H_{28}O_6$

hRf Value

(Figure 8-12)

The hRf values given are obtained from the following systems :

a)	Chloroform : Methanol	(8:2)	= 51
b)	Methanol : Benzene	(1:2)	= 41
c)	Methanol		= 75
d)	Acetone		= 56
e)	Ethyl acetate		= 21

Ultraviolet (UV) Absorption Spectrum (in Methanol)

(Figure 18)

λ max = 280 nm

Infrared (IR) Absorption Spectrum (KBr disc)

(Figure 19)

ν max = 3443, 2941, 1611 cm^{-1}

Nuclear Magnetic Resonance (NMR) Spectra

a) Proton (^{1}H) NMR Spectrum (in CD_3OD, 500 MHz)

(Figure 20-23)

See Table 4

b) Carbon (^{13}C) NMR Spectrum (in CD_3OD, 125.65 Mhz)

(Figure 24)

See Table 5

Table 4 ^{1}H-NMR assignment of ST-1

H position	δ (ppm)	multiplicity	J (Hz)
2	6.57	s	-
3-OCH_3	3.85 (3H)	s	-
5-OCH_3	3.37 (3H)	s	-
7	*2.56	dd	(14.8,11.3)
7	*2.71	dd	(14.8,4.58)
8	1.65	m	-
9	*3.50	dd	(10.9,4.88)
9	*3.57	dd	(4.88,4.89)
2′,6′	6.37 (2H)	s	-
3′,5′-OCH_3	3.73 (6H)	s	-
7′	4.30	d	10.49
8′	1.99	m	-
9′	3.50 (2H)	dd	(5.49,17.39)

Table 5 ^{13}C-NMR Assignment of ST-1

Carbon position	Chemical shift (δ,ppm)
2	107.83
3	149.04
4	139.36
5	148.71
6	134.59
7	33.61
8	40.95
9	66.8
1′	126.30
2′	106.94
3′	147.75
4′	138.94
5′	147.75
6′	106.94
7′	48.0
8′	42.4
9′	64.2
*3-OCH_3	60.20
*5-OCH_3	
3′-OCH_3	56.81
5′-OCH_3	56.70

* The assignment may be either one of these.

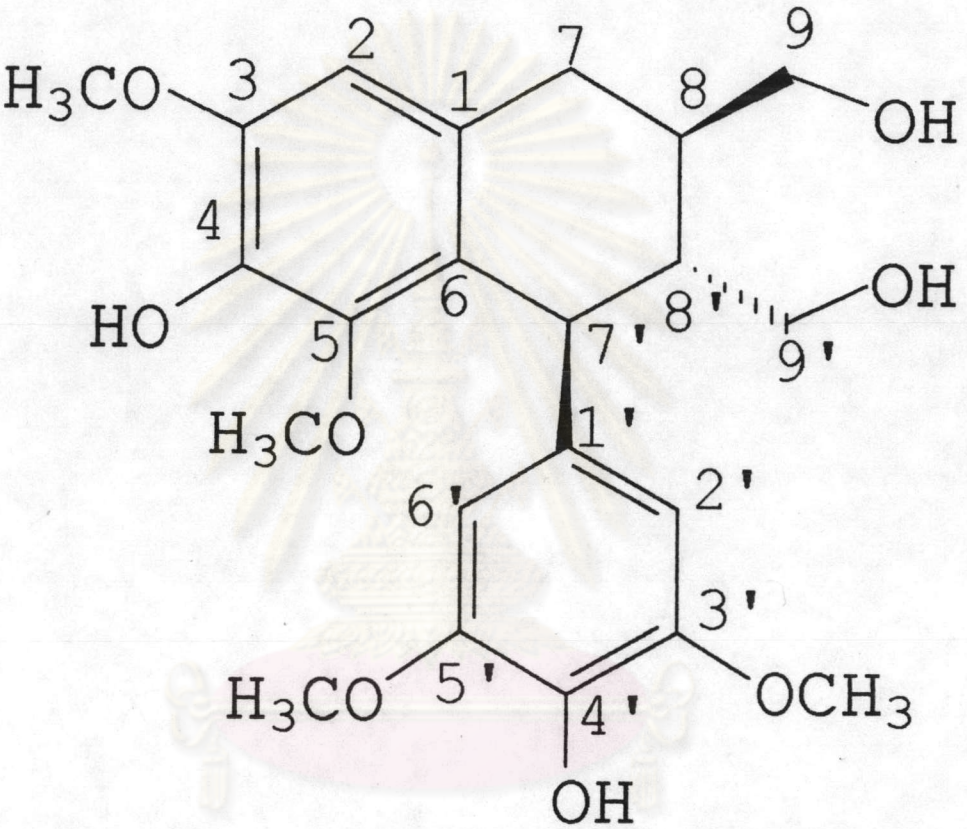
Mass Spectrum (EIMS)

(Figure 25-26)

m/z (% relative intensity)

420 (M+,48.57), 402 (31.89), 390 (2.56), 371 (10.76), 339 (4.44), 307 (3.82), 301 (4.18), 248 (10.42), 217 (17.83), 205 (22.60), 183 (25.51), 167 (28.95), 44 (100.00)

These data are in agreement with the published values of lyoniresinol, the known lignan isolated from *Ulmus thomasii* Sarg. of family Ulmaceae.[141] It is therefore concluded that ST-1 is lyoniresinol.



LYONIRESINOL

Characterization of ST-2

ST-2 was obtained as yellow needle crystals. It is soluble in chloroform . It gave orange color with dragendorff's reagent.

Melting Point

128-129 °c (decomposed)

hRf Value

(Figure 13-17)

The hRf values given are obtained from the following systems :

a)	Petroleum ether : Acetone	(7:3)	= 15
b)	Chloroform : Acetone	(9:1)	= 20
c)	Hexane : Ethyl acetate	(2:1)	= 8
d)	Chloroform : Methanol	(15:1)	= 60
e)	Hexane : Acetone	(1:1)	= 35