

CHAPTER III
EXPERIMENTAL



Source of plant materials

The leaves of *Uncaria macrophylla* Wall. were obtained from Khao-Yai National Park, Nakorn-Rachasima, Thailand in March, 1980. The plant was identified by Miss Ampai Yongboonkird, Head of Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

General techniques

1. The extraction of alkaloids

The dried coarsely powdered leaves were moistened with strong ammonium hydroxide solution overnight and were macerated with ethyl acetate for 7-10 days and filtered. The filtrate was evaporated under reduced pressure to dryness, mixed with glacial acetic acid and poured into warm distilled water to make 5% acetic acid solution. This solution was filtered and the filtrate was made alkaline (pH 9-10) with strong ammonium hydroxide solution and extracted with chloroform until negative test with Dragendorff's reagent was obtained. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated under reduced pressure to yield dry crude alkaloids.

2. Thin-layer chromatography

Technique : one way, ascending

Adsorbent : silica gel G(E.Merck) 30 g./60 ml. distilled water,
aluminium oxide G(E.Merck) 70 g./85 ml. distilled water.

Plate size : 10 cm. x 20 cm. and 20 cm. x 20 cm.
Layer thickness : 250 μ
Activation : air dried for 15 minutes and at 105°C for 1 hour.
Solvent system : 1) silica gel G/chloroform:acetone 5:4
2) silica gel G/chloroform:ethyl alcohol 95:5
3) silica gel G/anaesthetic diethyl ether
4) silica gel G/anaesthetic diethyl ether:ethyl acetate
1:1
5) silica gel G/ethyl acetate
6) alumina G/anaesthetic diethyl ether

Distance : 15 cm.

Temperature : 20°-30°C

Detection : a) Dragendorff's spray reagent

solution A : bismuth subnitrate(850 mg.), distilled water(40 ml.) and acetic acid(10 ml.)

solution B : potassium iodide(8 g.) and distilled water(20 ml.)

Solution A and B, 5 ml. each, were mixed, 20 ml. of glacial acetic acid and 70 ml. of the distilled water were added and used as spray reagent.

b) 0.2 M anhydrous ferric chloride in 35%w/v perchloric acid spray reagent.

Plate, after spraying was warmed gently with hot air stream from a hair dryer for 15 minutes.

3. Column chromatography

Column size : 1.5 cm.x 30 cm. and 2.5 cm.x 40 cm.

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

aluminium oxide neutral, activity III 0.063-0.200 mm. (E.Merck)

Packing : adsorbent pack dry into the column.

Addition of alkaloidal material to column :

crude alkaloid was dissolved in 2-3 ml. of organic solvent, mixed with small quantity of adsorbent, air dried, triturated and added onto the top of a dry column.

Solvent : anaesthetic diethyl ether, ethyl acetate, ethyl alcohol

Collection of eluate : fractions of 10 ml. were collected.

Examination of eluate : Those fractions giving an orange-red colour with Dragendorff's spray reagent were examined by thin-layer chromatography. The liked fractions were combined and evaporated under reduced pressure to dryness.

4. Physical constant

Melting points were determined by Reichert melting point apparatus. The values recorded are uncorrected.

5. Spectroscopy

a. Ultraviolet absorption spectra were obtained with a Unicam SP 1800 Spectrophotometer.

b. Infrared absorption spectra were obtained with a Perkin-Elmer 283 Spectrophotometer.

c. Nuclear magnetic resonance spectra were obtained with an HA-100 instrument in deuteriochloroform using tetramethylsilane($T_4M_4S_4$)

as internal reference.

d. Mass spectra were determined on an AEI MS 902 spectrometer at 70 eV with inlet temperature between 220°-240°C.

e. Circular dichroism spectra were obtained with Jasco J400X spectrometer.

The extraction and isolation of alkaloids from the leaves of *Uncaria macrophylla* Wall.

1. The extraction of crude alkaloids

The dried coarsely powdered leaves (3 kg) were moistened with strong ammonium hydroxide solution and allowed to stand overnight. The moistened powdered leaves were then macerated with ethyl acetate (7 L) for seven days and filtered. The marc was remacerated with ethyl acetate (7 L) and filtered. The filtrate was evaporated under reduced pressure to dryness, mixed and stirred thoroughly with glacial acetic acid (80 ml). It was then poured into warm distilled water to give 5% acetic acid solution (1.6 L) and left to stand overnight. The acidic solution was filtered through kieselguhr and made alkaline, pH about 9 to 10, with strong ammonium hydroxide solution. The alkaline solution was extracted with chloroform (16x250 ml) until negative test with Dragendorff's reagent was obtained. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated under reduced pressure. It was dissolved in ether (300 ml) to yield two portions of crude alkaloids, the ether-soluble (29.6 g) and the ether-insoluble alkaloid (0.5 g). Both were shown to have the same pattern in thin-layer chromatograms, which indicated that at least five alkaloids were present, without the addition of base-line alkaloids.

2. The isolation of alkaloids.

2.1 The isolation of alkaloid S₁

Crude alkaloid (1.5 g) was dissolved in chloroform (2 ml) and mixed with small amount of alumina activity III. It was dried, triturated and packed onto the top of dry alumina column(2.5 cm.x 40 cm.). The column was first eluted with anaesthetic diethyl ether (150 ml) and the eluate was collected in 10 ml. fractions. These fractions were observed using alumina G / anaesthetic diethyl ether TLC system and found that :-

the first fraction (25 ml) contained no alkaloid,

the second fraction (75 ml) contained an heteroyohimbine alkaloid, designated as S₁ (30 mg) and was subsequently identified as dihydrocorynantheine, and

the third fraction (50 ml) contained no alkaloid.

The crude alkaloid was then eluted with redistilled ethyl acetate and ethyl alcohol. Both were dried under reduced pressure to give similar alkaloids, Fraction I (1.2 g), shown to contain four oxindole alkaloids designated as S₂, S₃, S₄ and S₅ respectively.

2.2 The isolation of oxindole alkaloids from Fraction I

Fraction I (300 mg) was dissolved in chloroform (2 ml) and mixed with small amount of silica gel, dried and packed onto the top of dry silica gel column (2.5 cm.x 40 cm.). The column was eluted with redistilled ethyl acetate, 10-20 ml fractions were collected until negative test of alkaloid was obtained. The ethyl acetate fractions were observed by TLC and the liked fractions were combined. It was concentrated under reduced pressure to dryness to give the following fractions:-

- a) first fraction (15 ml) contained an oxindole alkaloid designated as S₂.
- b) second fraction (60 ml) contained two oxindole alkaloids, S₂ and S₃, or Fraction A.
- c) third fraction (200 ml) contained alkaloid S₂, S₃ and S₄ and was called Fraction B.
- d) fourth fraction (500 ml) contained alkaloid S₂, S₃ and S₅ and was called Fraction C.

From TLC examination, these four alkaloids are oxindoles, open E ring alkaloids which give blue to pink colour after spraying with 0.2 M. ferric chloride in 35 % w/v perchloric acid and warm in hot air stream about 15 minutes.

2.2.1 The isolation of alkaloids S₂ and S₃

Fraction A (100 mg) was dissolved in chloroform (2 ml) and mixed with small amount of silica gel. It was dried and packed onto the top of dry silica gel column (1.5 cm.x 30 cm.). The column was eluted with redistilled ethyl acetate and 5-10 ml fractions were collected. TLC examination was made and the liked fractions were combined.

The first fraction (15 ml) was shown to contain oxindole alkaloid, S₂, (15 mg). Bulking of the liked fractions from ten columns yielded 140 mg of S₂.

The second fraction (50 ml) contained alkaloids S₂ and S₃ which was the same as Fraction A.

The third fraction (50 ml) contained alkaloid S₃ (15 mg).

The dried first and third fractions were separately removed the impurities by using silica gel column chromatography using anaesthetic diethyl ether as eluant. The Dragendorff-negative impurities were eluted out first and followed by the pure oxindole, open E ring alkaloid S₂ and S₃ respectively.

The alkaloids S₂ and S₃ were subsequently identified as corynoxine and isorhynchophylline respectively.

2.2.2 The isolation of alkaloid S₄

Fraction B (300 mg) was dissolved in 2-3 drops of chloroform, mixed with 25 ml of ethyl acetate : diethyl ether (1:3) and allowed to stand in the dark for 48 hours. White prism crystals of alkaloid S₄ were obtained and they were washed with diethyl ether. It was subsequently identified as corynoxine B.

2.2.3 The isolation of alkaloid S₅

Fraction C (300 mg) was dissolved in 2-3 drops of chloroform, mixed with 25 ml of ethyl acetate : diethyl ether (1:3) and allowed to stand for 36 hours in the dark. White needle crystals of alkaloid S₅ were obtained and washed with diethyl ether. S₅ was subsequently identified as rhynchophylline.

Identification of the isolated alkaloids.

The isolated alkaloids were identified by comparison of the hRf values, melting points, ultraviolet, infrared, nuclear magnetic resonance, mass and circular dichroism spectra with the data of reference alkaloids.

The hRf values given are those obtained with the following systems : -

- a) silica gel G / chloroform : acetone (5:4)
- b) silica gel G / chloroform : ethyl alcohol (95:5)
- c) silica gel G / ethyl acetate : anaesthetic diethyl ether (1:1)
- d) silica gel G / ethyl acetate : isopropyl alcohol : strong solution of ammonium hydroxide (100:2:1)
- e) alumina G / anaesthetic diethyl ether

1. Identification of S₁ as dihydrocorynantheine

S₁ was obtained as pale yellow amorphous. It was soluble in anaesthetic diethyl ether, ethyl acetate, chloroform and ethyl alcohol.

hRf values

a. 66 b. 45 c. 59 d. 75 e. 87

Melting point

76°-78°C (amorphous)

Molecular weight

368 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{\max} 232 nm., 283 nm.(sh.), 290 nm.

λ_{\min} 256 nm.

Infrared absorption spectrum (Potassium bromide)

ν_{\max} 3380 (NH-2° amine), 3080 (CH-aromatic),

2930 (CH-aliphatic), 1705 (ester carbonyl),

1635 (double bond), 1110 (ether), 735 (aromatic) cm^{-1}

NMR spectrum in deuteriochloroform at 100 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	0.82 (3h,d)	C(19)-CH ₃
	3.69 (3H,s)	vinyl-OCH ₃
	3.78 (3H,s)	ester-OCH ₃
	6.87-7.50 (4H,m)	aromatic-H
	7.33 (1H,s)	olefinic
	8.10 (1H,s)	imino

Mass spectrum

m/e (%) 368(M⁺,100), 367(77.1), 353(60.0), 311(11.4), 251(11.4),
239(14.3), 225(11.4), 223(2.7), 197(11.4), 184(74.3),
170(40.0), 169(40.0), 156(34.3).

S₁ is identical in hRf values, melting point, ultraviolet, infrared, NMR and mass spectra with identified dihydrocorynantheine spectral data from *Mitragyna parvifolia* (Roxb.)Korth. (Shellard *et al.*, 1969a; Beckett *et al.*, 1969). It is therefore concluded that S₁ is dihydro-corynantheine.

2. Identification of S₂ as corynoxine

S₂ was obtained as yellow amorphous from anaesthetic diethyl ether. It was soluble in anaesthetic diethyl ether, chloroform, ethyl acetate.

hRf values

a. 71 b. 46 c. 61 d. 69 e. 45

Melting point

153°-155°C (amorphous)

Molecular weight

384 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol) λ_{\max} 212 nm., 243 nm., 283 nm. (sh.) λ_{\min} 223 nm.Infrared absorption spectrum (Potassium bromide)

ν_{\max} 3180 (imino), 3080 (CH-aromatic), 2960 (CH-aliphatic),
 1705 (ester/oxindole carbonyl), 1620 (double bond),
 1110 (ether), 750 (aromatic) cm^{-1}

NMR spectrum in deuteriochloroform at 100 MHz in δ values (ppm)
from tetramethylsilane (T.M.S.)

δ	0.81	(3H, d)	C(19)-CH ₃
	3.46	(3H, s)	vinyl-OCH ₃
	3.54	(3H, s)	ester-OCH ₃
	6.80-7.32	(4H, m)	aromatic-H
	7.34	(1H, s)	olefinic
	7.32-7.52	(d)	C(7)-A series
	8.73	(1H, s)	imino

Mass spectrum

m/e (%) 384(M⁺, 100), 369(4.2), 367(4.2), 353(8.3), 239(37.5),
 238(25.0), 224(20.8), 210(12.5), 208(16.7), 146(12.5),
 145(8.3), 144(12.5), 130(20.8), 69(29.2).

Circular dichroism spectrum(concentration 326.6 $\mu\text{g}/5 \text{ ml.}$ Methyl alcohol)

cotton effect (C.E.)

wave length \rightarrow 200-251 nm. : positive C.E.

- 251-293 nm_o : negative C_oE_o

- 293-325 nm_o : positive C_oE_o

S₂ is identical in hRf values, melting point, ultraviolet, infrared, NMR, C_oD_o and mass spectra with identified corynoxine spectral data (Trager *et al.*, 1968a; Beckett *et al.*, 1969; Phillipson and Hemingway, 1973c, 1975). It is therefore concluded that S₂ is corynoxine.

3. Identification of S₃ as isorhynchophylline

S₃ was obtained as white amorphous. It was soluble in anaesthetic diethyl ether, ethyl acetate, chloroform and ethyl alcohol.

hRf values

a. 68 b. 41 c. 55 d. 67 e. 44

Melting point

68-70°C (amorphous)

Molecular weight

384 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 214 nm_o, 244 nm_o, 283 nm_o (sh.)

λ_{min} 224 nm_o

Infrared absorption spectrum (Potassium bromide)

ν_{max} 3260 (imino), 2920 (CH-aromatic), 2850 (CH-aliphatic),
1705 (ester/oxindole carbonyl), 1630 (double bond),
1110 (ether), 750 (aromatic) cm⁻¹

NMR spectrum in deuteriochloroform at 100 MHz in δ values (ppm)

from tetramethylsilane (T_oM_oS_o)

δ 0.80 (3H, s) C(19)-CH₃

3.59 (3H,s)	vinyl-OCH ₃
3.69 (3H,s)	ester-OCH ₃
6.8-7.32 (4H,m)	aromatic-H
7.34 (1H,s)	olefinic
7.32-7.53 (d)	C(7)-A series
8.10 (1H,s)	imino

Mass spectrum

<i>m/e</i> (%)	384(M ⁺ ,100), 369(5.6), 367(5.6), 353(11.1), 239(50), 238(33.3), 224(27.8), 210(16.7), 208(16.7), 146(16.7), 145(16.7), 144(11.1), 130(16.7), 69(44.4).
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Circular dichroism spectrum

(concentration 309 µg/ 5 ml. Methyl alcohol)

cotton effect (C.E.)

wave length	- 200-241 nm. : positive C.E.
	- 241-293 nm. : negative C.E.
	- 293-327 nm. : positive C.E.

S₃ is identical in hRf values, melting point, ultraviolet, infrared, NMR, C.D. and mass spectra with identified isorhynchophylline spectral data (Trager *et al.*, 1968a; Beckett *et al.*, 1969; Shellard *et al.*, 1971; Phillipson and Hemingway, 1975). It is therefore concluded that S₃ is isorhynchophylline.

4. Identification of S₄ as corynoxine B

S₄ was obtained as white prism crystal. It was very soluble in chloroform, slightly soluble in ethyl acetate, ethyl alcohol and insoluble in anaesthetic diethyl ether.

hRf values

a. 48 b. 32 c. 37 d. 56 e. 20

Melting point

171°-172°C

Molecular weight

384 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{\max} 214 nm_a, 246 nm_a, 284 nm_a (sh.)

λ_{\min} 224 nm_a

Infrared absorption spectrum (Potassium bromide)

ν_{\max} 3250 (imino), 3130 (CH-aromatic), 2960 (CH-aliphatic),
1720 (ester carbonyl), 1695 (oxindole carbonyl),
1645 (double bond), 1110 (ether), 740 (aromatic).

NMR spectrum in deuteriochloroform at 100 MHz in δ values(ppm)from tetramethylsilane(T₄M₄S₄)

δ	0.90 (3H,d)	C(19)-CH ₃
	3.58 (3H,s)	vinyl-OCH ₃
	3.62 (3H,s)	ester-OCH ₃
	6.73-7.35 (4H,m)	aromatic-H
	7.28-(1H,s)	olefinic
	8.86 (1H,s)	imino

Mass spectrum

m/e (%) 384(M⁺,100), 369(7.7), 367(7.7), 353(7.7), 239(76.9),
238(30.7), 224(46.2), 210(15.4), 208(23.1), 146(23.1),
145(15.4), 144(30.7), 130(46.2), 69(46.2).

Circular dichroism spectrum(concentration 213 $\mu\text{g}/3\text{ ml.}$ Methyl alcohol)

cotton effect (C.E.)

wave length - 200-232 nm. : negative C.E.

- 232-330 nm. : positive C.E.

S_4 is identical in hRf values, melting point, ultraviolet, infrared, NMR, C.D. and mass spectra with identified corynoxine B spectral data (Trager *et al.*, 1968a; Beckett *et al.*, 1969; Phillipson and Hemingway, 1973c, 1975). It is therefore concluded that S_4 is corynoxine B.

5. Identification of S_5 as rhynchophylline

S_5 was obtained as white needle crystal. It was soluble in chloroform, slightly soluble in ethyl acetate, ethyl alcohol and insoluble in anaesthetic diethyl ether.

hRf values

a. 35 b. 26 c. 12 d. 31 e. 7

Melting point

198°-200°C

Molecular weight

384 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol) λ_{max} 217 nm., 244 nm., 284 nm. (sh.) λ_{min} 224 nm.Infrared absorption spectrum (Potassium bromide)

ν_{max} 3180 (imino), 3080 (CH-aromatic), 2960 (CH-aliphatic),
1730 (ester carbonyl), 1705 (oxindole carbonyl),
1640 (double bond), 1100 (ether), 740 (aromatic)

NMR spectrum in deuteriochloroform at 100 MHz in δ values(ppm)
from tetramethylsilane(T.M.S.)

δ	0.84 (3H,d)	C(19)-CH ₃
	3.61 (3H,s)	vinyl-OCH ₃
	3.72 (3H,s)	ester-OCH ₃
	6.85-7.24 (4H,m)	aromatic-H
	7.26 (1H,s)	olefinic
	8.82 (1H,s)	imino

Mass spectrum

$m/e(\%)$	384(M ⁺ ,100), 369(8.3), 367(8.3), 353(8.3), 239(83.3), 238(33.3), 224(25), 210(16.7), 208(16.7), 146(8.3), 145(8.3), 144(8.3), 130(16.7), 69(33.3)。
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Circular dichroism spectrum

(concentration 238 $\mu\text{g}/3\text{ ml}$ Methyl alcohol)

cotton effect (C.E.)

wave length	- 200-221 nm. : negative C.E.
	- 221-250 nm. : positive C.E.
	- 250-262 nm. : negative C.E.
	- 262-330 nm. : positive C.E.

S₅ is identical in hrf values, melting point, molecular weight, ultraviolet, infrared, NMR, C.D. and mass spectra with identified rhynchophylline spectral data (Trager *et al.*, 1968a ; Beckett *et al.*, 1969; Shellard *et al.*, 1971 ; Phillipson and Hemingway, 1975). It is therefore concluded that S₅ is rhynchophylline.