Chapter III

Experimental

I. Source of Plant Materials

The barks of *Neolitsea aureo-sericea* Kosterm. were obtained from Pakthongchai, Nakorn-Rachasima, Thailand in July, 1985 by Mr. Anun Sonngai, Research & Training Unit in Re-Afforestation Project, Pakthongchai, Nakorn-Rachasima. This plant was identified by Mr. Weerachai Na Nakorn, Forest Herbarium, Royal Forest Department and Mrs. Jaree Bunsiddhi, Botany Section, Department of Medical Science. The voucher specimen is deposited at the Royal Forestry Department, Phahonyothin Road, Bangkok, Thailand.

II. General Technique

A. Thin layer chromatography (TLC)

Technique : One way, ascending, tank saturated

Adsorbent : Silica gel G (E. Merck) 30 g/60 ml of distilled water

Plate size : 10 cm x 20 cm, 20 cm x 20 cm

Layer thickness : 250 μ, 500 μ

Activation : Air dried for 15 minutes and then at 110°C for 45 minutes.

Solvent systems : a) Silica gel G/hexane : acetone (1:1)
b) Silica gel G/benzene + ethyl acetate + methanol (8 + 4 + 2)
c) Silica gel G/ethyl acetate : methanol (8:2)
d) Silica gel G/ethyl acetate : methanol (1:1)
e) Silica gel G/chloroform : methanol (95:5)
f) Silica gel G/chloroform : methanol (8:2)

Distance : 15 cm.

Temperature : 25-30°C

Detection : Modified Dragendorff's spray reagent, according to Munier and Macheboenfs.

Solution A : Bismuth subnitrate (850 mg), distilled water (40 ml), and glacial 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 distilled water were added and used as spray reagent.

The alkaloids give orange or yellowish orange spots with the reagent.

: Iodine Vapour

Green and brown spots developed after treating the plate in iodine vapour for 2-10 minutes.

B. Column chromatography

Column size : 2.5 cm x 40 cm, 5 cm x 50 cm.

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

Packing : Adsorbent packed wet into the column

Addition of alkaloidal material to column :

The portion of crude alkaloid was dissolved in small amount of chloroform : methanol (1:1), then mixed with small quantity of the adsorbent, then dried, triturated and added to the top of the column.

Solvent : Hexane AR J.T. Baker

: Benzene E. Merck.

: Chloroform G.R. Merck.

: Ethyl acetate E. Merck.

: Methanol G.R. Merck.

Collection of eluate :

Fractions of 20 ml were collected.

Examination of eluate : Those fractions giving green or brown colour by exposure to iodine vapour and orange-red colour with Modified Dragendorff's spray reagent were examined by thin-layer chromatography. The like fractions were combined and evaporated under reduced pressure to dryness.

C. Physical constants

Melting points : Melting points were determined by Gallen kamp MFB 595 melting point apparatus. The values recorded was uncorrected.

Optical rotations : Optical rotations were determined by Perkin-Elmer 241 polarimeter.

D. Spectroscopy

Ultraviolet absorption spectrum :

The ultraviolet absorption spectra (UV) were determined by double beam spectrophotometer, Hitachi model 220A.

Infrared absorption spectra :

The infrared absorption spectra (IR) were determined in potassium bromide disc by Shimadzu infrared spectrophotometer, model IR-440, absorption bands were reported in wave number (cm^{-1}).

Nuclear magnetic resonance spectra :

Nuclear magnetic resonance (NMR) spectra were determined by Bruker WP 200 MHz. Supercon (FT) spectrometer. Tetramethylsilane (TMS) was used as an internal standard and dimethyl sulfoxide (DMSO) as solvent. For 60 MHz NMR spectra, deuterochloroform ($CDCl_3$) was used as solvent. The chemical shifts were reported on the ppm scale.

Mass spectra : Mass spectra (MS) were determined by Kratos MS 9/50 instrument, in ionization voltage 70 ev and ionization current 300 μA.

III. Extraction and isolation of alkaloids from the barks of *Neolitsea aureo-sericea* Kosterm.

A. Extraction of crude alkaloid

The dried coarsely powdered bark (3 kg) of *Neolitsea aureo-sericea* Kosterm. was extracted in a Soxhlet apparatus with petroleum ether (b.p. 60-80°C) for 48 hours and the petroleum ether extract was filtered. The filtrate was concentrated under reduced pressure to give brownish oily mass (8.5 gm). The defatted bark was extracted exhaustively with 95% ethyl alcohol by maceration in portions at room temperature. The combined filtrate was concentrated under reduced pressure to sticky mass

and dried in vacuum desiccator (311.005 gm). The crude ethyl alcohol extract was divided into two equal portions, the glacial acetic acid (50 ml) was added to each portion (approx. 150 gm) and then poured into warm distilled water in order to give about 5% acetic acid solution. The acid solution was filtered through kieselguhr layer and made alkaline, pH about 8 to 9, with strong ammonia solution. The alkaline solution was extracted with chloroform until it gave faint positive test with Modified Dragendorff's reagent. The combined chloroform extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to give dried yellowish-brown crude alkaloid NAA (31.351 gm). The aqueous layer was further extracted with chloroform : methanol 2:1 until nearly exhausted. The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure to give brown crude alkaloid NAB (10.52 gm).

B. Isolation of alkaloids

The crude alkaloid NAA (31.045 gm) was divided into six equal portions. Each portion (approx. 5 gm) was separated by silica gel (250 gm) column chromatography in the same manner. Dissolved each portion of crude alkaloid NAA in mixture chloroform : methanol (1:1), mixed with small amount of silica gel. After the solvent was evaporated, triturated and add to the top of the silica gel column (12.5 cm x 50 cm) which was wet packed before by using chloroform : methanol (95:5) as solvent. The fractions were collected 50 ml each. The like fractions as shown in Table 3 and after TLC examination were combined together,

each was concentrated and named alphabetically from A-F.

(Figure 9, page 138)

Table 3 Inofrmation of the crude alkaloid NAA column chromatographic isolation.

Fraction Number	Solvent	Combined fraction	Remarks
	chloroform:methanol		
1 - 22	95:5	A	no lakaloid
23 - 31	95:5	B	alkaloid NA-1
32 - 37	95:5	C	alkaloid NA-1, mixture of alkaloids NA-2 and NA-4
38 - 45	95:5	D	mainly alkaloid NA-1, mixture of alkaloids NA-2, NA-3 and NA-4
46 - 58	95:5	E	mixture of alkaloids NA-2, NA-3 and NA-4
59 - 78	95:5	F	alkaloids NA-2, NA-4 and traces of alkaloid NA-3

The fraction A gave negative test with Modified Dragendorff's reagent and was not further investigated.

1. Isolation of alkaloid NA-1

The fraction B was shown by TLC to contain only one spot of alkaloid and traces of impurity. It was purified by re-column chromatography (silica gel/chloroform). The fractions of pure alkaloid were combined together and concentrated under reduced pressure, then crystallized from methanol. It yielded pale pink plates (0.1797 gm), designated as NA-1.

2. Isolation of alkaloids NA-2, NA-3 and NA-4

The fraction E was shown by TLC to contain mixture of alkaloids NA-2, NA-3 and NA-4. Fraction E (2.179 gm) was isolated by column chromatography (silica gel/benzene + ethyl acetate + methanol, 8+ 4 + 2), collecting in 20 ml fractions. The like fractions were combined in accordance with the TLC pattern and named alphabetically from Ea-Ef as shown in Table 4 (Figure 10, page 139)

Table 4 Information of Fraction E column chromatographic isolation.

Fraction number	Solvent	Combined fraction	Remarks
	benzene + ethyl acetate + methanol		
1 - 30	8 + 4 + 2	Ea	no alkaloid
31 - 37	8 + 4 + 2	Eb	alkaloid NA-3
38 - 85	8 + 4 + 2	Ec	mixture of alkaloids NA-2, NA-3 and NA-4
86 - 100	8 + 4 + 2	Ed	mixture of alkaloids NA-2, NA-4 and traces of NA-3
101 - 125	8 + 4 + 2	Ee	mixture of alkaloids NA-2 and NA-4
126	methanol	Ef	no alkaloid

2.1 Isolation of alkaloid NA-3

The portion of fraction Eb (0.06 gm) was shown by TLC (silica gel G/benzene + ethyl acetate + methanol, 8 + 4 + 2) to contain two substances at the same Rf value. One was an alkaloid, another was impurity which fluoresced blue under the UV light. It was purified by re-column chromatography (silica gel/hexane : acetone, 1:1). The fractions of pure alkaloid were combined together and concentrated under reduced pressure, then crystallized from hexane. It yielded colourless needles (0.012 gm), designated as NA-3. (Figure 11, page 140)

2.2 Isolation of alkaloids NA-2 and NA-4

The portion of fraction Ee (0.21 gm) was shown by TLC (silica gel G/benzene + ethyl acetate + methanol, 8 + 4 + 2) to contain mixture of alkaloids NA-2, NA-4 and trace of impurity which fluoresced blue under UV light. It was purified by preparative thin layer chromatography on silica gel G using ethyl acetate and methanol (1:1) as a solvent. There were three bands of separated substances. Only substances of the second band and of the third band were recovered seperately from the scraped-off zones by mixture of chloroform and methanol (1:1). The chloroform-methanol extract was evaporated to dryness under reduced pressure. The substance from the second band was designated as NA-2 (0.0261 gm) and the third band as NA-4 (0.0097 gm). (Figure 12, page 141)

IV. Characterization and Identification of Isolated Isoquinoline Alkaloids

Identification of the alkaloids were deduced from the data of hRf values, melting points, specific optical rotations, ultraviolet, infrared, nuclear magnetic resonance and mass spectra.

The hRf values were obtained from the following solvent systems :-

a) Silica gel G/chloroform : methanol (17:3)

b) Silica gel G/chloroform : methanol (9:1)

c) Silica gel G/benzene + ethyl acetate + methanol (8 + 4 + 2)

d) Silica gel G/acetone : methanol (3:2)

e) Silica gel G/ethyl acetate : methanol (1:1)

f) Silica gel G/methanol

1. Identification of Alkaloid NA-1 as Isoboldine

NA-1 was obtained as pale pink plates from methanol and gave positive test with Modified Dragendorff's reagent. It was soluble in chloroform, in ethyl acetate and slightly soluble in methanol.

1.1 hRf Values

The hRf values were obtained from the solvent systems as mentioned before.

a = 48.3, b = 51.6, c = 34.8, d = 61.8, e = 51.3, f = 67.1

(hRf = ratio between the distances of the spot on chromatogram and the solvent front, multiplied by 100)

The thin layer chromatograms of alkaloid NA-1 are shown in Figures 13-18, page 138-147.

1.2 Melting Point

121-122°C.

1.3 Specific Optical Rotation

$[\alpha]_D^{25^oC}$ +53.65 (c = 0.0233 g/ml, chloroform)

1.4 Ultraviolet Absorption Spectrum

λ_{max} ($CHCl_3$) : 240, 280, 312, 320 nm

λ_{max} ($CHCl_3$ + 0.1 N HCl) : 240, 280, 312, 320 nm

λ_{max} ($CHCl_3$ + 0.1 N NaOH) : 240, 280, 312, 320 nm

1.5 Infrared Absorption Spectrum (KBr disc)

ν_{max} 760, 810, 1020, 1078, 1105, 1230, 1280, 1462, 1510, 1600, 2910, 3180 cm^{-1}

1.6 Nuclear Magnetic Resonance Spectrum (60 MHz, $CDCl_3$ and TMS as internal standard)

δ(ppm)	2.52 (3H, S)	$N-CH_3$
	3.90 (6H, S)	2 x OCH_3
	6.52 (1H, S)	C_3-H
	6.80 (1H, S)	C_8-H
	8.02 (1H, S)	$C_{11}-H$

1.7 Mass Spectrum

m/e (% rel int.) 327(M^+, 98), 326(100), 312(29), 310(12), 296(10), 284(44), 269(12), 253(22), 252(15)

NA-1 was identical in UV, IR, NMR, and mass spectra with isoboldine, the known alkaloid isolated from *Neolitsea fuscata* (Thwait.) Alston (Gunatilaka *et al.*, 1981). It was therefore concluded that NA-1 is isoboldine.

2. Identification of Alkaloid NA-2 as Bisnorargemonine

NA-2 was obtained as white amorphous powder (0.0261 gm) from chloroform. It was soluble in chloroform, in ethyl acetate and in methanol.

2.1 hRf Values

The hRf values were obtained from the solvent systems as mentioned before

a = 33.1, b = 36.6, c = 21.3, d = 61.1, e = 50.0, f = 67.1

The thin layer chromatograms of alkaloid NA-2 are shown in Figures 13-18, pages 138-147.

2.2 Melting Point

238-239°C (amorphous powder)

2.3 Specific Optical Rotation

$[\alpha]_D^{25^oC}$ -214.29 (c = 0.0049 g/ml, methanol)

2.4 Ultraviolet Absorption Spectrum

λ_{max} ($CHCl_3$) 288, 292 nm

λ_{max} ($CHCl_3$+ 0.1 N HCl) : 285, 290 nm

λ_{max} ($CHCl_3$+ 0.1 N NaOH): 288, 292 nm

2.5 Infrared Absorption Spectrum (KBr disc)

ν_{max} 760-780, 1260, 1420, 1480, 1510, 1610, 2910, 3500 cm^{-1}

2.6 Nuclear Magnetic Resonance Spectrum (200 MHz, DMSO and TMS as internal standard)

δ(ppm)	2.32	(3H, S)	$N-CH_3$
	3.63	(3H, S)	$O-CH_3$
	3.69	(3H, S)	$O-CH_3$
	6.30	(1H, S)	$C_{10}-H$
	6.43	(1H, S)	C_4-H
	6.50	(1H, S)	C_1-H
	6.65	(1H, S)	C_7-H

2.7 Mass Spectrum

m/e (% rel. int.) 328 (M^++1, 11), 327(M^+, 53), 326(44), 312(7), 191(24), 190(100), 177(4), 176(3), 175(10)

The UV, IR, NMR and Mass spectra of NA-2 were identical with data from the literature of bisnorargemonine isolated from *Argemone hispida* Gray, *A. munita* Dur. & Hilg. subsp. *rotundata* (Rydb.) G.B. Ownb, *Eschscholzia* species and *Thalictrum dasycarpum* Fisch. (Stermitz and Seiber, 1966; Soine and Kier, 1963; Slavik and Slavíková, 1970; and Kupchan and Yoshitake, 1968). It was therefore concluded that NA-2 is bisnorargemonine.

3. Identification of NA-3 as Norcinnamolaurine

NA-3 was obtained as colourless needles (0.012 gm) from hexane and gave positive test with Modified Dragendorff's reagent. It was soluble in acetone, in chloroform, in ethyl acetate and in methanol.

3.1 hRf Values

The hRf values were obtained from the solvent systems as mentioned before.

a = 39.7, b = 42.5, c = 41.3, d = 57.8, e = 58.4, f = 65.1

The thin layer chromatograms of alkaloid NA-3 are shown in Figures 13-18, page 138-147.

3.2 Melting Point

196-197°C.

3.3 Specific Optical Rotation

$[\alpha]_D^{25^oC}$ +90.92 (c = 0.0055 g/ml, ethanol)

3.4 Ultraviolet Absorption Spectrum

λ_{max} ($CHCl_3$) 242, 288 nm

λ_{max} ($CHCl_3$ + 0.1 N HCl) 240, 285 nm

λ_{max} ($CHCl_3$ + 0.1 N NaOH) 240, 288 nm

3.5 Infrared Absorption Spectrum (KBr disc)

ν_{max} 600, 1020, 1240, 1460, 1480-1500, 1600, 2900, 3410 cm^{-1}.

3.6 Nuclear Magnetic Resonace Spectrum (200 MHz, DMSO and TMS as internal standard)

δ(ppm)	5.90 (2H, S)	$-OCH_2O-$
	6.59 (1H, S)	C_8-H
	6.67 (2H, d, J = 8.445)	C_2,-H, C_3,-H
	6.79 (1H, S)	C_5-H
	7.04 (2H, d, J = 8.405)	C_5,-H, C_6,-H
	9.17 (1H, S)	NH

3.7 Mass Spectrum

m/e (% rel. int.) 283(M^+, 0.31), 282(1.67), 177(33), 176(100), 146(4), 118(9), 107(12), 91(11), 77(8), 65(7)

NA-3 was identical in UV, IR, NMR and Mass spectra with norcinnamolaurine, the known alkaloid isolated from *Cinnamomum* sp. (Gellert and Summons, 1980). It was therefore concluded that NA-3 is norcinnamolaurine.

4. Identification of Alkaloid NA-4 as (+)-Reticuline

NA-4 was obtained as yellow amorphous powder (0.0097 gm) and gave positive test with Modified Dragendorff's reagent. It was soluble in chloroform, in ethyl acetate and in methanol.

4.1 hRf Values

The hRf values were obtained from the solvent systems as mentioned before.

a = 26.5, b = 31.8, c = 21.3, d = 41.4, e = 41.6, f = 46.7.

The thin layer chromatograms of alkaloid NA-4 are shown in Figures 13-18, pages 138-147.

4.2 Melting Point

125-126°C. (amorphous powder)

4.3 Specific Optical Rotation

$[\alpha]_D^{25^\circ C}$ = +55.00 (c = 0.0001 g/ml, ethanol)

4.4 Ultraviolet Absorption Spectrum

λ_{max} ($CHCl_3$) 241, 283 nm

λ_{max} ($CHCl_3$ + 0.1 N HCl) 240, 290 nm

λ_{max} ($CHCl_3$ + 0.1 N NaOH) 241, 282 nm

4.5 Infrared Absorption Spectrum

ν_{max} 600, 700, 1020, 1130, 1220-1280, 1440, 1520, 1600, 2900, 3410 cm^{-1}.

4.6 Nuclear Magnetic Resonance Spectrum (200 MHz, DMSO and TMS as internal standard)

δ(ppm)	2.49 (3H, S)	$N-CH_3$
	3.85 (6H, S)	2 x OCH_3
	6.35 (1H, S)	C_8-H
	6.54 (1H, S)	C_5-H
	6.59 (2H, dd, J_o= 8.32, J_m= 1.73)	$C_{2'}$-H
	6.74 (1H, d, J_o= 8.32)	$C_{3'}$-H
	6.75 (1H, dd, J_m= 1.73)	$C_{6'}$-H

4.7 Mass Spectrum

m/e (% rel. int.) 329(M^+, 17), 328(64), 327(60), 326(36), 312(87), 193(29), 192(100), 177(39)

The UV, IR, NMR and Mass Spectra of NA-4 were identical with data from the literature of (+)-reticuline isolated from *Thalictrum revolutum* Tops (Wu, Beal, Wu and Doskotch, 1977). It was therefore concluded that NA-4 is (+)-reticuline.