CHAPTER III

EXPERIMENTAL

Source of plant material

The plant material of *Strychnos minor* Dennst. was collected from Krabi Province, Southern part of Thailand, during March 1989. It was authenticated by comparison with the herbarium specimens at Royal Forest Department, Bangkok, Thailand; and was authenticated as the form of *S. silvicola* A.W.Hill by comparison with the herbarium specimen at Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

A voucher specimen of the plant material has been deposited in the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

General techniques

1. Chromatographic techniques

1.1 Thin-layer chromatrography (TLC)

Technique	:	One way ascending
Adsorbent	:	Silica gel G (E. Merck) 30 g in 60 ml distilled water
Plate sizes	:	5 x 20 cm, 10 x 20 cm or 20 x 20 cm

Layer Thickness	:	0.25 mm
Activation	:	Air-dried for 15 minutes and then warm in hot air oven at 110 ° C for 1 hour
Solvent system	:	various solvent systems depending on materials
Distance	:	15 cm
Laboratory temperature	:	28-35 ° C
Detection	:	1) UV light (254 and 366 nm) 2) Ferric chloride-perchloric acid 3) Dragendorff´s reagent

1.2 Preparative thin-layer chromatography (PLC)

Technique	:	One way ascending
Adsorbent	:	Mixture of silica gel G (E. Merck) and silica gel GF_{254} (E. Merck) in 2:1 ratio
Plate size	:	20 x 20 cm
Layer thickness	:	0.50 mm
Solvent system	:	MeOH:Benzene (10:1)
Distance	:	20 cm
Laboratory temperature	:	28-35 ° C

Detection : UV light (254 and 366 nm)

Substance recovering : The scraped off zones were warmed with a mixture of $CHCl_3$:MeOH (1:1), and filtered. After removal of the solvent, the residues were taken in $CHCl_3$ and filtered.

1.3 Spraying reagent

1) Ferric chloride-perchloric acid

This reagent was made by mixing 1 ml 0.5 M ferric chloride soluton with 100 ml 35% aqueous perchloric acid solution.

The reagent gave a range of colours depending on the nature of the subsitution pattern in the aromatic part of the *N*-acylindoline nucleus and also on the difference type of alkaloid skeletons. The colours would either develop immediately after spraying or only after heating the chromatographic plates at 90°C for 5 to 30 minutes.

2) Dragendorff's reagent

This reagent was used as a general alkaloidal detecting reagent which characterized the alkaloids by giving orange colour.

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

The working solution was made by mixing 10 ml of stock solution with 20 ml glacial acetic acid and 70 ml distilled water.

2. Column chromatography (CC)

Column sizes	:	The glass columns 3/4 - 4 inches in diameter were used depending on the quantity of sample to be separated.
Adsorbent	:	Silica gel 60 (E. Merck)
Packing method	:	Wet packing
Solvent	:	Various solvent systems depending on materials

3. Melting point

Melting point were determined on a Gallenkamp Melting Point Apparatus Model MFB 595.

The melting points were uncorrected.

4. Spectroscopy

4.1 Ultraviolet (UV) spectroscopy

Ultraviolet absorption spectra were determined on a Hitachi SP-150-20 double beam.

4.2 Infrared (IR) spectroscopy

Infrared absorption spectra were recorded as KBr disc on a Perkin Elmer Model 283 Spectrophotometer. The absorption bands were reported in wave number (cm^{-1}).

4.3 Nuclear Magnetic Resonances (NMR) Spectroscopy

The ^{1}H NMR and ^{13}C NMR spectra were obtained with three different instruments :

4.3.1 Nuclear Magnetic Resonance spectrometer Model FX 90 (JEOL) (90 MHz) for A-1 and AA-1.

4.3.2 Nuclear Magnetic Resonance spectrometer Model Varian XL-300 (300 MHz) for A-1.

4.3.3 Nuclear Magnetic Resonance spectrometer Model Bruker (300 MHz) for A-1 and AA-1.

Tetramethylsilane was uses as an internal standard (δ = 0.00).

4.4 Mass spectroscopy (MS)

The low resolution mass spectra were obtained on a Mass spectrometer Model DX 300 (JEOL) with direct inlet system operating at 70 ev, the temperature range between 150° to 300°.

5. Lyophilization

Some pure solution were dried by Termovac Lyophilizer.

6. Solvent

The solvents of commercial grade were redistilled before used.

Extraction

The dried and ground stem (4.8 Kg) was basified with 500 ml conc. ammonium hydroxide and left overnight. The basified material was macerated for 5 days with chloroform (10 x 18 l). The combined chloroform extracts were concentrated under reduced pressure. The chloroform extract (99 g) was further dissolved in a small volume of chloroform and then extracted with 5% sulfuric acid (20 x 200 ml). The combined acid extracts were basified with conc. ammonium hydroxide and then extracted with chloroform (20 x 200 ml).

The combined chloroform extracts were washed with a small volume of water, dried over anhydrous sodium sulphate, and then evaporated to dryness under reduced pressure to yield a gold-yellow syrupy mass of crude tertiary alkaloids 13 g (0.27%).

Separation procedure

The crude tertiary alkaloids (12.7 g) was chromatographed over silica gel 60 (1000 g) and then eluted with MeOH:Acetone:EtOAc (4:2:1) and MeOH. Fractions were collected and combined as portions according to the result of TLC check.

portions	eluent		weight (g)
A	MeOH:Acetone:EtOAc (4:2:1)	960 ml	0.0000
B	"	280 ml	0.2272
C	"	1200 ml	2.9922
D	"	1400 ml	0.5983
E	"	2000 ml	1.0612
F	"	2960 ml	4.9647
G	"	1800 ml	0.3285
	MeOH	1680 ml	
H	"	7360 ml	0.8335

Isolation of the chemical substances from various portions

Portion A

The TLC of portion shown no substance and was not investigated.

Portion B

This portion was not further investigated due to the small amount of substance obtained.

Portion C

This portion contained mainly a sterol and many alkaloids, some of which gave gray-brown and pink colours with $FeCl_3$-$HClO_4$ reagent. The sterol was isolated as a pure compound by using colume chromatography, but it was not the substance to be interested. Whereas the alkaloidal content in this portion was very low and a lot of dirty mass was included. This portion was not further investigated.

Portion D

This portion contained at least 7 alkaloids. Four of which gave gray-brown colour with $FeCl_3$-$HClO_4$ reagent. Two of them gave pink-violet colour with the same reagent. The remaining one was a yellow base which gave more intense yellow colour to $FeCl_3$-$HClO_4$ reagent. Because of the small amount and some dirty mass included, this portion could not to be separated as pure alkaloids.

Portion E

This portion contained at least 5 yellow alkaloidal bases which gave more intense yellow colour with $FeCl_3$-$HClO_4$ reagent. Because the decomposition taken place during the process of purification, this portion was not further investigated.

Portion F

This portion was dissolved in methanol and filtered. The methanol insoluble residue was collected and further crystallized in a mixture of chloroform-acetone to yield prismatic crystals of alkaloid A-1 (1.03 g).

The methanol filtrate was evaporated and was later subjected to column chromatography for the isolation of the residual alkaloid A-1 as well as other alkaloids.

1. The first column chromatography

The residual material from portion F (2.5 g) was chromatographed over silica gel 60 (200 g) and eluted with Benzene:MeOH:NH_4OH (30:10:1). Seventy-two fractions (40 ml each) were collected after loading.

Thin layer chromatography of the materials from the combined fractions indicated that the alkaloid A-1 was found as major compound in fraction 17-23 and 24-26. While the other combined fractions contained serveral alkaloids which gave orange, brown, pink or violet and at

least 2 yellow bases with $FeCl_3$-$HClO_4$ reagent. But unfortunately the material was too small amount to be further investigated and some alkaloids decomposed to a dirty mass during purification.

2. The second column chromatography

The combined fraction 17-23 from the first column (800 mg) was sujeccted to column chromatography with silica gel (80 g), and eluted with Benzene:Acetone:H_2O (3:11:1). Forty-three fractions (20 ml each) were collected.

The combined fraction 14-43 (750 mg) was dried by using vacuum evaporator and followed by lyophilizer to give a solid residue. Crystallization of the residue in chloroform-acetone mixture yielded prismatic crystals of alkaloid A-1 (54 mg).

The combined fraction 8-11 (46.5 mg) appeared to contain a clean yellow base. But the base readily decomposed to a dirty mass during purification by preparative thin-layer chromatography and they were not further investigated.

3. The third column chromatography

The combined fraction 24-26 (180 mg) from the first column was chromatographed over silica gel (20 g), and eluted with MeOH:Benzene:H_2O (4:1:1). Twenty-four fractions (20 ml each) were collected.

The combined fraction 16-24 (52.5 mg) was dried under reduced pressure followed by crystallization in chloroform-acetone mixture to give prismatic crystals of alkaloid A-1 (48.5 mg).

Portion G

As the same as portion F, the major alkaloid in this portion was alkaloid A-1. So the material was dissolved in methanol and filtrated. The methanol insoluble mass was collected and further crystallized in chloroform-acetone mixture to give prismatic crystals of alkaloid A-1 (110 mg).

The methanol filtrate was further subjected to column chromatography for the isolation of residue A-1 and other alkaloids.

1. The first column chromatography

The residual material from portion G (300 mg) was chromatographed over silica gel (30 g), and eluted with $CHCl_3$:MeOH (6:1). Eighty fractions (30 ml each) were collected.

The combined fraction 13-17 indicated the presence of alkaloid A-1 and it was crystallized from chloroform-acetone mixture (70 mg).

Another interesting combined fraction was fractions 37-54. Thin-layer chromatography of this combined

fractions showed a major alkaloid which gave gray-brown colour with $FeCl_3$-$HClO_4$ reagent. This alkaloid was further investigated by using second column chromatography.

2. The second column chromatography

The combined fraction 37-54 (273 mg) from the first column chromatography was chromatographed over silica gel (30 g), and eluted with MeOH:Benzene (10:1). Thirty fractions (20 ml each) were collected.

The interesting alkaloid was detected almost pure in fraction 13-15, but the crystallization of this compound was unsuccessful. So the whole material was evaporated under reduced presure to dryness to give pale yellow colour amorphous powder (77 mg). This alkaloid was coded AA-1.

Moreover, the alkaloid AA-1 was detected in fractions 16-18 with other substances. It was further isolated by using preparative thin-layer chromatography.

3. Preparative thin-layer chromatography

The combined fraction 16-18 (31.5 mg) from the second colume of portion G was subjected to the preparative thin-layer chromatography in solvent MeOH:Benzene (10:1). Four times developing were carried out.

The chromatogram gave two zones of suubstances. The lower zone, fluoresced under short wave ultraviolet light and was not further investigated, while the upper zone was the zone of alkaloid AA-1. The purified alkaloid was crystallized as colourless amorphous powder in chloroform-acetone mixture (21.5 mg).

Portion H

This portion contained at least four alkaloids which gave pink-violet colour with $FeCl_3$-$HClO_4$ reagent. But it was characterized as a syrupy mass. So the material was dissolved in methanol and filtered. The dirty methanol insoluble material gave negative test to Dragendorff's reagent and it was discarded.

The methanol soluble material was evaporated under reduced pressure to dryness (747 mg) and subjected to further investigated by using colume and preparative thin-layer chromatography. Some fractions gave mixture of crystals which can not isolated, while some others contained too small amount of bases to be isolated.

Characterization of the isolated alkaloids

Two alkaloids were isolated from the stem of *Strychnos minor* Dennst. The chemical and physical characteristic properties of the individual alkaloids are described as follows :

A-1

The base crystallized from chloroform-acetone as colourless prismatic crystals. It gave grayish-brown colour with the ferric chloride-perchloric acid spraying reagent after warming with hot air. The colour changed to chocolate-brown after 30 minutes at 90°. Its total yield is 1.3125 g (0.027%).

hRf values

MeOH:Benzene:H_2O (4:1:1) = 14.57

MeOH:Acetone:EtOAc (4:2:1) = 16.67

MeOH:Benzene:NH_4OH (10:30:1) = 54.55

MeOH:$CHCl_3$ (1:5) = 23.61

Melting point

206-207 ° C

Ultraviolet absorbtion spectrum (see figure 16, page 168)

(in MeOH) λ_{max} 209.9, 222.2, 255.2, 289.2 nm

(in MeOH + NaOH)

λ_{max} 204.0, 220.2, 256.3, 295.9 nm

Infrared absorption spectrum (KBr disc)

ν_{max} (cm^{-1}) : 3480, 3200, 2950, 2700, 1635, 1600, 1580, 1460, 1415, 1380, 1320, 1240, 1140, 1080, 1010, 980, 950, 920, 880, 860, 840, 800, 760, 750, 710, 680, 650, 600, 580, 550 (see figure 17, page 169)

Mass spectrum (EIMS) (see figure 18, page 170)

m/z (% rel. int) : 398 (100), 380 (28.1), 369 (30.0), 353 (41.8), 352 (25.7), 339 (26.1), 190 (65.4), 180 (88.7), 176 (26.2), 174 (27.4), 162 (24.0), 134 (26.4), 43 (32.7)

Proton nuclear magnetic resonance spectrum

(300 MHz, $CDCl_3$) (see figure 19, page 171)

chemical shift (ppm)	proton	multiplicity
10.18	1H	singlet
6.73	1H	doublet
6.57	1H	doublet
5.85	1H	triplet
5.30	1H	doublet
4.84	1H	doublet-doublet
4.22	1H	doublet
3.87	3H	singlet
3.86	1H	singlet
3.70	1H	doublet
3.68	1H	doublet-doublet
3.40	1H	singlet
3.34	1H	doublet-doublet
2.81	1H	multiplet
2.72	1H	doublet
2.45	3H	singlet
2.21	1H	triplet of doublet
1.92	1H	doublet-doublet
1.72	1H	doublet-doublet-doublet
1.57	1H	triplet of doublet
1.40	1H	triplet of doublet

Carbon-13 nuclear magnetic resonance spectrum

(22.5 MHz, $CDCl_3$) (see figure 20, page 172)

chemical shift (ppm)	multiplicity
172.654	singlet
150.225	singlet
143.020	singlet
137.982	singlet
131.246	singlet
130.018	singlet
126.334	doublet
112.141	doublet
110.407	doublet
93.667	doublet
66.905	doublet
58.887	doublet
56.612	quartet
55.203	triplet
53.416	triplet
53.036	singlet
51.574	triplet
45.885	doublet
38.193	triplet
28.712	doublet
25.191	triplet
22.861	quartet

AA-1

The base was obtained as white amorphous powder. It gave grayish-brown coloured with the ferric chloride-perchloric acid spraying reagent after warming at 90°C. Its total yield is 98.5 mg (0.002%).

hRf values

MeOH:Benzene:H_2O (4:1:1) = 36.11

MeOH:Acetone:EtOAc (4:2:1) = 11.11

MeOH:Benzene:NH_4OH (10:30:1) = 27.27

MeOH:$CHCl_3$ (1:5) = 6.94

Ultraviolet absorption spectrum (see figure 21, page 173)

(in MeOH) λ_{max} 235.0, 250.8, 282.3, 318.5 nm

Infrared absorption spectrum (KBr disc)(see figure 22, page 174)

ν_{max} (cm^{-1}) : 3440, 2950, 1635, 1610, 1460, 1440, 1380, 1250, 1080, 1015, 920, 860, 800, 715, 640

Mass spectrum (EIMS) (see figure 23, page 175)

m/z (% rel. int) : 429 (0.59), 412 (0.84), 398 (8.40), 396 (12.10), 380 (37.80), 378 (15.32), 367 (29.79), 349 (40.59), 190 (33.29), 189 (12.89), 180 (9.55), 176 (16.39), 174 (9.63), 149 (11.96), 135 (21.78), 121 (21.43), 119 (41.85), 44 (100), 43 (29.20)

Proton nuclear magnetic resonance spectrum (90 MHz, $CDCl_3$)

chemical shift (ppm)	proton	multiplicity
10.078	1H	singlet
7.266	2H	singlet
6.270	1H	ill-define triplet
5.302	1H	singlet
4.870	1H	doublet-doublet
4.348	1H	singlet
4.289	1H	doublet
3.875	6H	singlet
2.433	3H	singlet

(see figure 24, page 176)

Proton nuclear magnetic resonance spectrum (300 MHz, $CDCl_3$)

chemical shift (ppm)	multiplicity
10.07	singlet
6.77	doublet
6.74	doublet
6.25	triplet
4.86	doublet-doublet
4.37	singlet
4.28	doublet
4.13	doublet
3.94	doublet
3.9-4.0	multiplet
3.88	singlet
3.87	singlet
3.77	doublet-doublet
3.54	singlet
2.50	singlet
2.43	singlet
1.98	ill-define triplet of doublet
1.60	ill-define triplet of doublet
1.28	ill-define triplet of doublet
0.8-1.0	multiplet

(see figure 25, page 177)

Carbon-13 nuclear magnetic resonance spectrum

(22.5 MHz, $CDCl_3$) (see figure 26, page 178)

chemical shift (ppm)	multiplicity
172.810	singlet
151.000	singlet
138.069	singlet
136.758	singlet
133.720	doublet
129.548	singlet
125.615	singlet
112.326	doublet
110.479	doublet
92.420	doublet
81.760	doublet
71.980	triplet
69.300	triplet
65.846	doublet
56.550	ill-define quartet
54.167	singlet and ill-define triplet
46.539	doublet
34.800	triplet
29.318	ill-define
27.709	ill-define
23.061	ill-define quartet