CHAPTER III

EXPERIMENTAL

1. Source of plant materials

The leaves of *Anthocephalus chinensis* Achille Richard were obtained from Ayuthya, Thailand in April - May 1976. The plant materials were authenticated by comparison with the specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives.

2. General techniques

2.1 Thin layer chromatography (TLC)

The experimental details are summarised as follows :-

Analytical

Technique :	One way, ascending
Adsorbents :	a) Aluminium oxide G (E. Merck), calcium sulphate binder 10%, 70 g/80 ml of distilled water.
	b) Silica gel G (E. Merck), calcium sulphate binder 13%, 30 g/60 ml of distilled water.
Plate size :	20 cm x 10 cm
Layer thickness :	250 μ
Activation :	Air dried for 15 minutes and then at 105° C for 1 hour

Solvent systems : a) Silica gel G/Chloroform, ethyl alcohol 1+1

b) Silica gel G/Chloroform, methanol 6+4

c) Silica gel G/Butanol, acetic acid, water 4+1+1

d) Silica gel G/Ethyl acetate, isopropyl alcohol, 28% NH_4OH 40+7.5+2.5

e) Silica gel G/Chloroform, n-propyl alcohol, diethylamine 5+20+25

f) Silica gel G/Chloroform, ethyl alcohol, 1% NH_4OH 45+45+10

g) Silica gel G/ethyl acetate

h) Silica gel G/ethyl acetate, methanol, 25% NH_4OH 85+10+5

i) Aluminium oxide G/Chloroform, ethanol 1+1

Distance : 15 cm

Laboratory temperature : 24°-30° C

Detection of alkaloid on chromatographic plate :

Chromogenic reagents employed were :-

a) Dragendorff's spray reagent

b) Ferric chloride-perchloric acid reagent

c) Ceric sulphate reagent

Colours developed :-

a) The colour reaction giving an orange-red colour after spraying

b) & c) Plate, after spraying, was warmed in hot air oven. The colour reaction is blue green after heating then turning to greenish brown.

2.2 Column chromatography

Adsorbent : Aluminium oxide, neutral (E. Merck)

Packing of column : Absorbent packed dry into the column.

Addition of alkaloidal material to column :

Solution of alkaloid in small volume of volatile mixed with small quantity of adsorbent, air dried and added to the top of a dry column.

Solvents :

a) Anaesthetic diethyl ether (Macfarlan Smith Ltd; Edinburgh)
b) Anhydrous sodium sulphate (May & Baker)
c) Chloroform (I.C.I.)
d) Ethyl alcohol 95% (The Government Pharmaceutical Organisation)
e) Glacial acetic acid (May & Baker)
f) Methanol (E. Merck)
g) Strong solution of ammonium hydroxide (BDH Chemical Ltd., Poole, England)

Authentic samples : Cadambine tetra-acetate and 3α-dihydrocadambine penta-acetate kindly supplied by Dr. R.T. Brown, Dept. of Chemistry, The Victoria University of Manchester, Manchester, England.

2.3 Physical constants

Melting points : Melting points were determined by Büchi melting point apparatus. Results were uncorrected.

Optical rotation : Specific rotation were determined by Dr. R.T. Brown, Dept. of Chemistry, The Victoria University of Manchester, England.

Microanalysis : Elemental analysis was determined in the Department of Science, Ministry of Industry.

2.4 Spectroscopy

Ultraviolet absorption spectrum

Ultraviolet absorption spectrum was recorded in ethanol using a Unicam SP 1800 recording spectrophotometer in Department of Food Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Infrared absorption spectra

Infrared absorption spectra were obtained in Nujol mull (pure liquid paraffin) and potassium bromide disc by Perkin-Elmer 421 Grating Spectrophotometer in Department of Science, Ministry of Industry.

Nuclear magnetic resonance (NMR) spectra

NMR spectra were determined in CD_3OD at 60 MH_z using Perkin-Elmer R12 instrument of The Department of Medical Science, Ministry of Public Health, and in $CDCl_3$ by Dr. R.T. Brown, Department of Chemistry, The Victoria University of Manchester,

Manchester, England.

3. The isolation of alkaloid from the leaves of *Anthocephalus clinensis* Achille Richard

3.1 Isolation of alkaloid

The dried coarsely powdered leaves (13.5 kg) were macerated with 95% ethyl alcohol (30 L) for three days and filtered. The marc was remacerated with another portion of ethyl alcohol (10 L). The combined filtrate was concentrated under reduced pressure to syrupy mass till no traces of ethyl alcohol left, mixed with glacial acetic acid (500 ml) then poured into a large volume of warm water to give about 5% acetic acid solution, well shaken and left to stand overnight. The filtered acid extract was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (40 x 500 ml). The combined chloroform extract was washed with distilled water (4 x 500 ml), dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield a syrupy crude base (16.8 g).

Crude based (16.8 g) was divided into 12 equal portions. Each portion was dissolved in chloroform (20 ml), mixed with small amount of aluminium oxide, let the content air-dried and placed onto the top of dry aluminium oxide column (2.5 x 40 cm).

The alkaloids were eluted with mixture of chloroform and ethyl alcohol, 1+1, the eluate was collected until no traces of alkaloid could be detected. This eluate was evaporated under reduced pressure

to yield a dark brown alkaloidal base (5.6 g).

The adsorbent (aluminium oxide) were taken out of the column, air-dried and extracted with 5% acetic acid solution (10 x 500 ml). The filtered acid solution was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (12 x 500 ml). The combined chloroform was washed and dried over anhydrous sodium sulphate, then evaporated to dryness under reduced pressure yielding light brown amorphous powder (3.5 g).

Purification of light brown amorphous powder

The amorphous powder (3.5 g) was washed with diethyl ether to yield a cream coloured amorphous powder, NR-1, m.p. 180°-181° C (3.31 g).

3.2 Acetate formation of NR-1

One hundred mg of NR-1 was dissolved in acetic anhydride (15 ml) and 5 drops of pyridine were added. The mixture was vigorously stirred for 24 hours at room temperature. The excess acetic acid anhydride and pyridine was evaporated to dryness under reduced pressure yielding a yellow amorphous powder, m.p. 201°-202° C (115 mg).

3.3 Test for alkaloid

Colour tests

Reagent	Colour change
Ehrlich's	reddish brown
Fröhde's	brown → violet → blue
Mandelin's	blue → brown
Marme's	brown
Marquis's	brown
Mayer's	white ppt
Mecke's	blue
Nitric acid conc.	golden yellow
Sulphuric acid conc.	reddish brown → green
Wagner's	black ppt

3.4 Test for glycoside

Solution of NR-1 in water was boiled with 20% sulphuric acid, cool and made alkaline with NaOH. The mixture of equal portions of Fehling solutions A, and B were added, a yellow up to orange-red precipitates owing to the content of glycoside, was produced after warming in boiling water bath indicated the presence of reducing sugar.

4. Characterisation

NR-1 is obtained as pale cream coloured amorphous powder (m.p. 180°-181° C). It is soluble in water, slightly soluble in ethyl alcohol, chloroform, and insoluble in ether.

NR-1 acetate derivative is a yellow amorphous powder (m.p. 201°-202° C). It is freely soluble in water, chloroform, or ethyl alcohol.

Thin layer chromatography

hRf value of NR-1 base on Silica gel G/Chloroform, ethyl alcohol, 1+1 = 70

hRf value of NR-1 base on Silica gel G/Chloroform, ethyl alcohol, 1% NH_4OH in water 45+45+10 = 66

hRf value of NR-1 base on Silica gel G/Chloroform, methanol, 6+4 = 66

hRf value of NR-1 base on Silica gel G/Butanol, acetic acid, water, 4+1+1 = 73

hRf value of NR-1 acetate on Silica gel G/Ethyl acetate = 57

Specific rotation

NR-1 base	$[\alpha]_D^{25}$	($CHCl_3$)	-40°
NR-1 acetate	$[\alpha]_D^{25}$	($CHCl_3$)	-137°

Utraviolet absorption spectrum of NR-1 in ethyl alcohol

λ_{max}^{EtOH} = 230 nm

The UV absorption was the sum of indole and β-alkoxyacrylate chromophore.

Infrared absorption spectrum of NR-1 base in KBr disc

ν max = 3370 cm^{-1} (indolic -NH, OH bond)

= 1630 cm^{-1} (double bond C = C)

= 2900 cm^{-1} (methyl CH_3)

= 1020 cm^{-1} (-C-O-C-)

Infrared absorption spectrum of NR-1 acetate in Nujol mull

ν max = 3340 cm^{-1} (indolic -NH)

= 1720 cm^{-1} (-C-)

= 1625 cm^{-1} (double bond C = C)

Microanalysis

found C 59.12%, N 5.11%, H 6.35%

calc. for $C_{27}H_{34}N_2O_{10}$ C 59.33%, N 5.12%, H 6.27%

The most decisive structural evidence was obtained from the NMR spectra of the alkaloid and its penta-acetate. It was possible to recognise that the spectra indicated that the substance was a monoglycoside of an indolic alkaloid of the expected molecular weight. The features associated with the indolic portion of the molecule, the methyl β-alkoxyacrylate, and the glycosidic part (acetylated in the case of the derivative) could be recognised. Correlations are available which give evidence that the latter feature is a β-glucoside. A detailed listing of these spectra are as follow :

NMR spectrum and assignments to NR-1 acetate in $CDCl_3$

Protons	Chemical shift (τ)	Multiplicity
1 (H)	2.21	broad
5, 6 (2H)	6.75-7.45	m
9, 10, 11, 12 (H)	2.50-2.98	m
(4-aromatic protons)		
17 (H)	2.61	s
21 (H)	4.50	d
$COOCH_3$ (3H)	6.26	s
OAc (sugar moiety)	7.91, 7.97, 7.99, 8.03	s

However, it is more clearly visible in the spectrum of the penta-acetate than in that of the free alkaloid.

NMR spectrum and assignments to NR-1 base in CD_3OD

Protons	Chemical shift (τ)	Multiplicity
3 (H)	6.12	s
5, 6 (2H)	6.7-7.4	broad
9, 10, 11, 12 (H)	2.4-3.01	m
(4-aromatic protons)		
17 (H)	2.39	s
21 (H)	4.38	d
$COOCH_3$ (3H)	6.12	s
sugar moiety	6.4-6.7	m

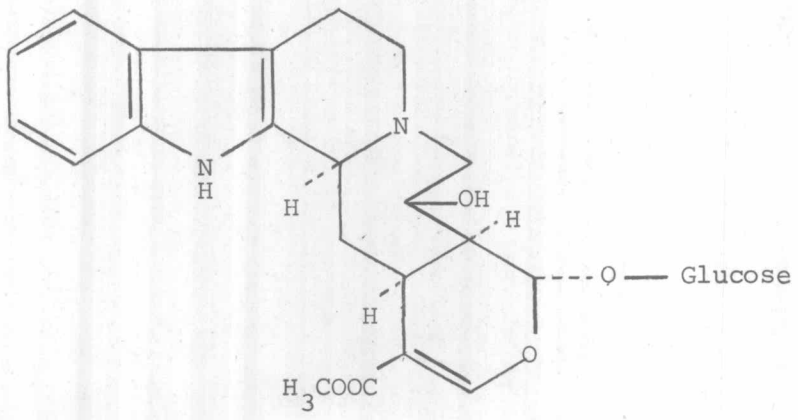
The terpenoid-derived portion is the heart of the structural problem. It can be noted that, since ring D is seven-membered, the stereochemical features based on chemical shifts are not as secure

as they would have been for a six-membered ring.

Circular dichroism

CD spectrum exhibited a strong positive Cotton effect between 250 nm and 300 nm indicating an α orientation of the alkyl residue at C-3.[32]

NR-1 has been identified by UV, IR, NMR, CD spectra, specific rotation, melting point, mixed melting point, microanalysis and hRf values on TLC along with authentic sample kindly supplied by Dr. R.T. Brown. It is therefore concluded that NR-1 is 3α-dihydrocadambine.



3α-dihydrocadambine