CHAPTER III

EXPERIMENTAL

1. Source and authentication of plant material

The leaves of *Uncaria attenuata* Korth. were collected from Chumporn, Thailand in August, 1975.

Authentication was undertaken by comparison with authentic specimens of the Rijksherbarium, Leiden, The Netherlands by Dr. C.E. Ridsdale.

2. General techniques

2.1 Extraction of alkaloids

The coarsely powdered plant material was moistened with 10 % ammonium hydroxide solution and macerated with ethyl alcohol. The filtered ethyl alcohol extract was concentrated to low bulk under reduced pressure, mixed with glacial acetic acid and poured into a large volume of warm distilled water to give about 5 % acetic acid solution. The filtrate was made alkaline (pH 9) with strong solution of ammonium hydroxide and extracted with chloroform. After washing with distilled water and drying over anhydrous sodium sulphate, this extract was evaporated under reduced pressure to yield crude alkaloidal extract.

2.2 Purification of crude alkaloidal extract

The crude alkaloidal extract was dissolved in small amount of chloroform and extracted with 5 % sulphuric acid solution. The acid solution was then made alkaline (pH 9) with strong solution of ammonium hydroxide and extracted with chloroform. The extracts were washed with distilled water, dried over anhydrous sodium sulphate, combined and evaporated to dryness under reduced pressure to yield the purified alkaloidal mixture.

2.3 Thin layer chromatography (T.L.C.)

2.3.1 Analytical

Technique : one way, ascending.

Adsorbents : a) aluminium oxide G (E. Merck), calcium sulphate binder 10 %; 50 g / 60 ml distilled water.

b) kieselgel G (E. Merck), calcium sulphate binder 13 %; 30 g / 60 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 then at 110° C for 1 hour.

Solvent systems : a) aluminium oxide G / chloroform

b) aluminium oxide G / ether

c) kieselgel G / chloroform

d) kieselgel G / chloroform : acetone (5 : 4)

e) kieselgel G / chloroform : ethyl alcohol (97 : 3)

f) kieselgel G / chloroform : ethyl alcohol (95 : 5)

g) kieselgel G / cyclohexane : ethyl acetate (1 : 1)

h) kieselgel G / ether

i) kieselgel G / ethyl acetate

j) kieselgel G / ethyl acetate : ether (1 : 1)

k) kieselgel G / ethyl acetate : isopropyl alcohol : strong solution of ammonium hydroxide (100 : 2 : 1).

Distance : 15 cm.

Laboratory temperature : 24° - 30° C.

Detection : a) ultraviolet light.

b) Dragendorff's spray reagent.

c) 0.2 M anhydrous ferric chloride in 35 % w/v perchloric acid spray reagent. Plate, after spraying, is warmed gently with a hot air stream for 15 minutes.

2.3.2 Preparative

Technique : one way, ascending.

Adsorbents : kieselgel G (E. Merck), calcium sulphate binder 13 % : kieselgel GF 254 (E. Merck), calcium sulphate binder 13 % (2 : 1), 120 g / 240 ml distilled water.

Plate size : 20 cm x 20 cm.

Layer thickness : 750 µ.

Activation : air dried overnight and then at 110° C for 1 hour.

Application : as a continuous streak using a capillary tube.

Solvent systems : a) diethyl ether

b) chloroform : ethyl alcohol (95 : 5).

Distance : 15 cm.

Laboratory temperature : 24^{o} - 30^{o} C.

Detection : ultraviolet light.

2.4 Column chromatography

Adsorbents : a) aluminium oxide, neutral (E. Merck).

b) kieselgel 0.040 - 0.063 mm (E. Merck).

Packing : adsorbents packed dry into the column.

Solvents : a) chloroform

b) cyclohexane

c) diethyl ether, anaesthetic

d) ethyl acetate

e) ethyl alcohol, 95 %

f) methyl alcohol.

2.5 Melting point

Melting point were determined by means of a Buchi melting point apparatus. The values recorded are uncorrected.

2.6 Ultraviolet absorption spectra

Ultraviolet absorption spectra were obtained with a Perkin Elmer 402 recording spectrophotometer.

2.7 Infrared absorption spectra

Infrared absorption spectra were obtained with Perkin Elmer 283 and 157G infrared spectrophotometers. The materials were examined in potassium bromide disc.

2.8 Nuclear magnetic resonance (N.M.R.) spectra

The N.M.R. spectra were obtained with a Perkin Elmer R 12A 60 MHz instrument using deuterochloroform solutions with tetramethylsilane (T.M.S.) as internal references.

^{13}C-N.M.R.. were obtained with a JEOL SP-100 spectrometer ^{13}C-N.M.R. & FT using deuterochloroform solutions with tetramethylsilane (T.M.S.) as internal references.

2.9 Mass spectra

The mass spectra were determined on AEI MS 902 mass spectrometer at 70 eV with inlet temperature between 210° and 240° C.

3. Isolation of individual alkaloids

3.1 Extraction of alkaloids from the leaves of *Uncaria attenuata* Korth.

Dried coarsely powdered leaves (5 kg) were moistened with 10 % ammonium hydroxide solution overnight and macerated with ethyl alcohol (14 L) for seven days and filtered. The marc was remacerated twice with other portions of ethyl alcohol (12 L and 10 L). The combined filtrate was concentrated under reduced pressure to a small volume. This concentrated solution yielded crystals (2.5 g) which was filtered off and designated as T_a. The mother liquor was then concentrated to a syrupy mass.

Glacial acetic acid (400 ml) was added to the syrupy mass, poured into warm distilled water (8 L) to give about 5 % acetic acid solution, well shaken and left to stand overnight. The filtered acid

extract was made alkaline (pH 9) with strong solution of ammonium hydroxide and extracted with chloroform (6 x 2 L). The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure to yield a brown crude extract (248 g).

The crude extract was dissolved in chloroform (200 ml) and extracted with 5 % sulphuric acid solution (10 x 300 ml). The acid solution was then made alkaline (pH 9) with strong solution of ammonium hydroxide and extracted with chloroform (10 x 400 ml). The extracts were washed with distilled water, dried over anhydrous sodium sulphate, combined and evaporated under reduced pressure to yield the purified alkaloidal extract designated as T_b (45.9 g) (Fig. XIX - XXII).

3.2 Separation of alkaloids by column chromatography

An aliquot (13 g) of the purified alkaloidal extract (T_b) was divided into four portions and each portion being dissolved in chloroform (5 ml), mixed with 2 g of aluminium oxide, air dried and added to a dry column of aluminium oxide (2.5 cm x 25 cm). It was eluted with anaesthetic diethyl ether (220 ml), chloroform (250 ml) and methyl alcohol (200 ml) respectively, 10 ml portions being collected. T.L.C. monitoring allowed bulking of like portions to give the following fractions :-

1. diethyl ether (100 ml) - containing no alkaloid.
2. diethyl ether (20 ml) - containing one alkaloid only, designated as TS_1 (0.04 g), which was subsequently identified as tetrahydroalstonine (Fig. XXIII - XXXI).

3. diethyl ether (100 ml) - shown by T.L.C. to contain two alkaloids, the eluate was evaporated to dryness under reduced pressure to give alkaloidal fraction T_c (0.81 g)(Fig. XXI-XXII).

   T_c was redissolved in dry diethyl ether and left to stand from which white crystals were deposited (0.65 g) designated as TS_2 which was subsequently identified as rauniticine. (Fig. XXIII - XXXI).

   The mother liquor, shown by T.L.C. to contain only one alkaloid with hR_f values equal to those of TS_1, was dried under reduced pressure to yield another batch of TS_1 (0.10 g) as an amorphous cream coloured alkaloid.

4. chloroform (250 ml) - shown by T.L.C. to contain at least four alkaloids. The eluate was evaporated to dryness under reduced pressure to yield alkaloidal fraction T_d (5.64 g). (Fig. XXI - XXII).

5. methyl alcohol (200 ml) - shown by T.L.C. to contain traces alkaloids in fraction T_d and base-line alkaloid(s). It was evaporated to dryness under reduced pressure. No further study has been made.

3.3 Isolation of alkaloids from the fraction T_d

The fraction T_d (1 g) was divided into two portions and each portion being dissolved in chloroform (5 ml), mixed with 2 g of kieselgel, air dried and added to a dry column of kieselgel (2.5 cm x 25 cm). It was eluted with cyclohexane : ethyl acetate, 1 : 1 (120 ml), chloroform : ethyl alcohol, 95 : 5 (150 ml) and methyl alcohol (100 ml)

respectively, 10 ml portions being collected. T.L.C. monitoring allowed bulking of like portions to give the following fractions :-

1. cyclohexane : ethyl acetate, 1 : 1 (40 ml) - containing no alkaloids.
2. cyclohexane : ethyl acetate, 1 : 1 (50 ml) - containing one alkaloid, designated as TS_3 (0.2179 g), which was subsequently characterised as 14-hydroxy-3-isoraunitidine (Fig. XXVI - XXXI).
3. cyclohexane : ethyl acetate, 1 : 1 (10 ml) - containing one alkaloid with hR_f values lower than those of TS_3, designated as TS_4 (0.004 g) (Fig. XXVI - XXXI).
4. cyclohexane : ethyl acetate, 1 : 1 (20 ml) - containing one alkaloid designated as TS_5 (0.016 g) (Fig. XXVI - XXXI).
5. chloroform : ethyl alcohol, 95 : 5 (150 ml) and
6. methyl alcohol (100 ml) - shown by T.L.C. to contain mixture of alkaloids. No further study of this fraction has been made.

3.4 Examination of alkaloids fraction T_a

T_a was shown by T.L.C. to contain three alkaloids. Two of them have hR_f values correspond to those of TS_1 and TS_2 which were identified as tetrahydroalstonine and raunitidine respectively. No attempt to isolate individual alkaloids has been made. The third alkaloid was an oxindole with hR_f values between those of TS_1 and TS_2 (Fig. XXXII - XXXIV). The amount of this alkaloid was too small to be isolated out.

4. Isomerisation of the isolated alkaloids

4.1 Isomerisation of 3-isorauniticine from rauniticine

Rauniticine (TS_2) (500.3 mg) was dissolved in glacial acetic acid (20 ml) containing mercuric acetate (1.8 g) and heated at 60°-65° C for 36 hours. The precipitated mercurous acetate was filtered off, hydrogen sulphide gas passed into the filtrate to remove excess mercuric ion and filtered again. Zinc dust (4 g) and distilled water (5 ml) were then added to the filtrate and stirred continuously, using magnetic stirrer, for 48 hours at room temperature. After filtration, the filtrate was made alkaline with strong solution of ammonium hydroxide and extracted with chloroform (3 x 20 ml). The chloroform extracts were washed with distilled water, dried over anhydrous sodium sulphate, combined and evaporated to dryness under reduced pressure (510.3 mg) to give alkaloidal fraction T_e. T.L.C. examination of alkaloidal fraction T_e indicated the presence of mainly two indolic spots, one with hR_f values corresponding to those of rauniticine.

An aliquot of T_e (107.8 mg) was dissolved in chloroform (2 ml) and streaked on preparative kieselgel G : kieselgel GF 254 (2 : 1) plates (750 μ thick, 20 cm x 20 cm) which were run in chloroform : ethyl alcohol (1 : 1). The appropriate zones were scraped off and extracted with chloroform : methyl alcohol, 1 : 1 (2×25 ml). Each combined extract was evaporated to dryness under reduced pressure and extracted with chloroform (2 x 25 ml). Evaporation under reduced pressure of the chloroform extract of one zone yielded rauniticine (44.9 mg), while that of another zone produced another indole compound (45.5 mg),

designated as TS_6 (Fig. XXXV - XXXVII). This compound was subsequently identified as 3-isorauniticine.

4.2 Isomerisation of akuammigine from tetrahydroalstonine

Tetrahydroalstonine (TS_1) (43.8 mg) was treated as described under 4.1, from which alkaloidal fraction T_f (28.5 mg) was obtained By using preparative T.L.C., kieselgel G : kieselgel GF 254 (2 : 1) plate, with diethyl ether as solvent, tetrahydroalstonine and another indole compound, designated as TS_7 were obtained separately (Fig. XXXV - XXXVII). This compound (TS_7) was subsequently identified as akuammigine.

5. Identification and characterisation

5.1 Identification of the isolated alkaloids and the isomerisation products

The alkaloids were identified by comparison of the hR_f values, melting points, ultraviolet, infrared, nuclear magnetic resonance and mass spectra with authentic alkaloids.

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

a) kieselgel G / chloroform : acetone, 5 : 4

b) kieselgel G / chloroform : ethyl alcohol, 95 : 5

c) kieselgel G / cyclohexane : ethyl acetate, 1 : 1

d) kieselgel G / diethyl ether

5.1.1 Identification of TS_1 as tetrahydroalstonine

TS_1 was obtained as rod crystals from diethyl ether. It was soluble in chloroform, ethyl alcohol and diethyl ether, and insoluble in light petroleum.

hR_f values

a) 82 b) 91 c) 85 d) 93

Melting point

210° C

Molecular weight

352 (mass spectrometry)

Ultraviolet absorption spectrum (Methyl alcohol)

λ_{max} 220, 283, 293 nm

λ_{min} 287 nm

Infrared absorption spectrum (Potassium bromide disc)

ν_{max} 3400 (imino), 1705 (ester and oxindole carbonyl), 1625 (double bond), 740 (benzene ring) cm^{-1}

Mass spectrum

m/e (%) 352 (M^+, 100), 351 (81), 337 (27), 323 (4), 321 (6), 293 (7), 265 (4), 251 (10), 225 (5), 223 (15), 221 (7), 209 (6), 197 (10), 184 (10), 170 (13), 169 (17), 156 (55), 143 (9), 130 (6).

Isomerisation

The isomerisation product (TS_7) of TS_1 was identified as akuammigine.

TS_1 is identical in hR_f values, melting point, ultraviolet, infrared and mass spectra with an authentic sample of tetrahydroalstonire from *Uncaria gambir* (Hunt.) Roxb. (Merlini *et al.*, 1970). In addition, TS_1 has been isomerised to akuammigine (*epiallo* isomer). It is therefore concluded that TS_1 is tetrahydroalstonine.

5.1.2 Identification of TS_7 as akuammigine

TS_7 was obtained as amorphous powder. It was soluble in chloroform, ethyl alcohol and diethyl ether, and insoluble in light petroleum.

hR_f values

a) 63 b) 64 c) 31 d) 43

Molecular weight

352 (mass spectrometry)

Ultraviolet absorption spectrum (Ethyl alcohol)

λ_{max} 228, 284, 296 nm

Infrared absorption spectrum (Nujol)

ν_{max} 3300 (imino), 1698 (ester carbonyl), 1680 (oxindole carbonyl), 1620 (double bond), 740 (benzene ring) cm^{-1}

TS_7 is identical in hR_f values, ultraviolet and infrared spectra with an authentic sample of akuammigine from *Mitragyna parvifolia* (Roxb.) Korth. (Shellard *et al.*, 1968a). It is therefore concluded that TS_7 is akuammigine.

5.1.3 Identification of TS_2 as raunitlcine

TS_2 was obtained as plate crystals from absolute ethyl alcohol. It was soluble in chloroform, ethyl alcohol, diethyl ether and ethyl acetate, and insoluble in light petroleum.

hR_f values

a) 42 b) 43 c) 33 d) 43

Melting point

215° C

Molecular weight

352 (mass spectrometry)

Ultraviolet absorption spectrum (Methyl alcohol)

λ_{max} 223, 284, 292 nm

λ_{min} 289 nm

Infrared absorption spectrum (Potassium bromide disc)

ν_{max} 3400 (imino), 1700 (ester and oxindole carbonyl), 1610 (double bond), 750 (benzene ring) cm^{-1}

N.M.R. spectrum in deuterochloroform at 60 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	1.47	(3 H, d, J 5.5 Hz)	C(19) - CH_3
	3.76	(3 H, s)	-OCH_3
	4.45	(1 H, br, m)	C(19) - H
	6.95 - 7.47	(4 H, m)	aromatic - H
	7.51	(1 H, s)	olefinic - H
	7.85	(1 H, s)	imino

Mass spectrum

m/e (%) 352 (M^+, 100), 351 (70), 337 (27), 323 (4), 321 (5), 293 (9), 265 (3), 251 (10), 225 (4), 223 (14), 221 (16), 209 (7), 197 (9), 184 (8), 170 (16), 169 (26), 156 (45), 143 (12), 130 (8)

Isomerisation

The isomerisation product (TS_6) of TS_2 was identified as 3-isorauniticine.

TS_2 is identical in hR_f values, melting point, ultraviolet, infrared, N.M.R. and mass spectra with an authentic sample of rauniticine kindly supply by Dr. M. Pais, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. In addition, TS_2 has been isomerised to 3-isorauniticine (*epiallo* isomer). It is therefore concluded that TS_2 is rauniticine.

5.1.4 Identification of TS_6 as 3-isorauniticine

TS_6 was obtained as amorphous powder. It was soluble in chloroform, ethyl alcohol and diethyl ether, and insoluble in light petroleum.

hR_f values

a) 68 b) 75 d) 55

Molecular weight

352 (mass spectrometry)

Infrared absorption spectrum (Potassium bromide disc)

ν_{max} 3350 (imino), 1690 (ester and oxindole carbonyl), 1620 (double bond), 735 (benzene ring) cm^{-1}

N.M.R. spectrum in deuterochloroform at 60 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ	1.38	(3 H, d)	C(19) - CH_3
	3.78	(3 H, s)	-OCH_3
	4.15	(1 H, m)	C(19) - H
	7.25	(4 H, m)	aromatic - H
	7.67	(1 H, s)	olefinic - H
	8.05*	(1 H, s)	imino

* disappears on deuteration

TS_6 is identical in hR_f values, infrared and N.M.R. spectra with an authentic sample of 3-isorauniticine kindly supply by Dr. M. Pais, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. It is therefore concluded that TS_6 is 3-isorauniticine.

5.2 Characterisation

Characterisation of TS_3 as 14-hydroxy-3-isorauniticine

TS_3 was obtained as amorphous powder. It was soluble in chloroform, ethyl alcohol, ethyl acetate, cyclohexane and diethyl ether, and insoluble in light petroleum.

hR_f values

a) 70 b) 88 c) 70 d) 77

Melting point

100° C

Molecular weight

368 (mass spectrometry)

CD spectrum

-ve Cotton effect at 285 nm

Ultraviolet absorption spectra

methyl alcohol : λ_{max} 244, 270, 279, 290 nm

methyl alcohol and hydrochloric acid :

λ_{max} 229, 270, 280, 290 nm

reversible with alkali

Infrared absorption spectrum (Potassium bromide disc)

ν_{max} 3440 (imino), 3350 (hydroxy), 2850, 2810, 2750 (Bohlmann bands), 1665 (ester carbonyl, H - bonded), 1620 (double bond) cm^{-1}

N.M.R. spectrum in deuterochloroform at 60 MHz in δ values (ppm) from tetramethylsilane (T.M.S.)

δ 1.42	(3 H, d, J 5.5 Hz)	C(19) - CH_3
3.80	(3 H, s)	-OCH_3
4.40	(1 H, q, J 6 and 12 Hz)	C(19) - H
5.95	(1 H, br, s)	C(14) - OH
7.25	(4 H, m)	aromatic - H
7.75	(1 H, s)	olefinic - H
9.35*	(1 H, br, s)	imino

* disappears on deuteration

^{13}C N.M.R. spectrum in deuterochloroform in δ values (ppm)

from tetramethylsilane

δ	171.843	s	-CO	76.420*	d C(14)
	156.723	d	C(17)	73.993*	d C(19)
	135.755	s	C(13)	65.159	d C(3)
	134.349	s	C(2)	55.355	t C(21)
	126.413	s	C(8)	52.928	t C(5)
	121.073	d	C(11)	52.054	q $-OCH_3$
	118.744	d	C(10)	37.712*	d C(15)
	117.699	d	C(9)	35.043*	d C(20)
	110.978	d	C(12)	21.792	t C(6)
	107.798*	s	C(7)	19.390	q C(18)
	105.080*	s	C(16)		

* possible interchangeable

Mass spectrum

m/e (%) 368 (M^+, 100), 167 (37), 353 (M^+-15, 13), 351 (M^+-17, 21), 350 (M^+-18, 57), 337 (9), 335 (10), 321 (11), 309 (19), 267 (21), 237 (13), 225 (36), 223 (30), 209 (21), 199 (21), 184 (24), 171 (56), 169 (63), 156 (40), 144 (30), 143 (26), 55 (40)

From the molecular weight, the CD, ultraviolet, infrared, N.M.R. and ^{13}C-N.M.R., and mass spectra obtained, TS_3 was, therefore characterised as 14-hydroxy-3-isorauniticine.