Chapter Three

Experimental

3.1 Source and Authentication of Plant Materials

Alstonia angustiloba Miq. (K680, UT3) was collected from Gua Musang, Kelantan (May 2008), and identification was confirmed by Dr. Richard C. K. Chung, Forest Research Institute of Malaysia (FRIM). The plant material was screened before any chemical analysis was carried out. Voucher specimen is deposited at the Herbarium, University of Malaya.

3.2 General

Melting points were taken on a hot stage Leitz-Wetzlar melting point apparatus and were uncorrected. Optical rotations were taken with Jasco P-1020 automatic digital polarimeter. UV spectra were obtained on a UV-3101 PC spectrometer in absolute ethanol. IR spectra were recorded on a Perkin-Elmer 1600 Series or a Perkin-Elmer RX1 FT-IR spectrophotometer. Mass spectra were obtained using Agilent 6530 Q-TOF mass spectrometer. ^{1}H NMR and ^{13}C NMR were recorded using JEOL JNM-LA 400 and ECA 400 spectrometers in $CDCl_3$ solution using tetramethylsilane (TMS) as the internal standard at 400 MHz and 100 MHz, respectively. X-ray diffraction analysis was carried out on a Bruker SMART APEX II CCD area detector system equipped with a graphite monochromator and a Mo Kα fine-focus sealed tube (λ = 0.71073 Å), 100. The structure was solved by direct methods (SHELXS-97) and refined with full-matrix least-squares on F^2 (SHELXL-97). All non-hydrogen atoms were refined anisotropically, and

all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters (I thank Mr Low Yun Yee for carrying out X-ray diffraction analysis). All solvents are of analytical grade and were distilled prior to use with the exception of diethyl ether which was passed through activated neutral alumina before use.

3.3 Chromatographic Methods

3.3.1 Column Chromatography

Column chromatography was performed using silica gel (9385, 540-63 mm, 230-240 Mesh ASTM). The ratio of silica gel to sample was approximately 30:1 for crude samples and 100:1 for semipure samples. The gel was made into slurry with chloroform before it was packed into the column and was allowed to equilibrate for at least an hour before use. The solvent systems normally used to elude the columns were chloroform with increasing methanol gradient or diethyl ether with increasing ethyl acetate gradient. Fractions were monitored by thin layer chromatography (TLC) and appropriate fractions were combined and where necessary subjected to further separation by re-chromatography or centrifugal TLC.

3.3.2 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was routinely used to detect and separate the various alkaloids. The crude alkaloidal extracts, fractions from chromatography, and isolated pure alkaloids were examined by TLC using pre-coated 5 cm x 10 cm plates, 0.25 mm thickness, silica gel 60 F_{254} (Merck, Darmstadt, GFR). The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated

chromatographic tanks with various solvent systems at room temperature. The alkaloidal spots were visualized by examination of the TLC plates under UV light (254 nm), followed by spraying with Dragendorff's reagent, which formed colour spots. The hR_f values of the alkaloids are tabulated in Table 3.1.

Table 3.1. The hR_f Values of Alkaloids Isolated from *A.angustiloba* Miq.

Alkaloids	**Solvent systems**				
	a	b	c	d	e
Angustilobine C (**1**)	0	4	0	15	13
Andransinine (**2**)	5	13	10	20	24
Angustiphylline (**3**)	5	7	2	23	12
19,20-*E*-Vallesamine (**4**)	4	11	7	22	19
17-*O*-Acetylvallesamine (**5**)	5	14	11	43	27
Yunnanensine (**6**)	5	4	0	13	8
Angustilobine A (**7**)	11	29	0	47	49
Angustilobine B (**8**)	3	5	2	20	9
Nor-6,7-*Seco*angustilobine B (**9**)	17	11	10	31	23
Nor-6,7-*Seco*-19,20α-epoxyangustilobine B (**10**)	4	5	2	23	25
Undulifoline (**11**)	2	4	0	15	7
Condylocarpine (**12**)	10	13	7	65	21
20*S*-Tubotaiwine (**13**)	6	5	6	26	14
N(4)-Demethylechitamine (**14**)	2	12	7	11	30
17-*O*-Acetyl-*N*(4)-demethylechitamine (**15**)	6	13	4	28	35
Alstolucine B (**16**)	10	7	1	33	20
Vincamine (**17**)	7	18	19	43	39
16*R*,19*E*-Isositsirikine (**18**)	3	8	3	8	15
Venoterpine (**19**)	4	28	18	24	42
Cantleyine (**20**)	2	57	39	33	68

Solvent systems:

a: Chloroform

b: Ethyl acetate

c: Diethyl ether

d: Methanol : Chloroform (1 : 20)

e: Methanol : Ethyl acetate (1 : 10)

3.3.3 Preparative Centrifugal Thin Layer Chromatography

Centrifugal Preparative Layer Chromatography was carried out using a round chromatographic plate measuring 24 cm in diameter with the action of a centrifugal force to accelerate mobile phase flow across the circular plate. To prepare the chromatographic plate, the edge of the plate was secured with cellophane tape to form a mould. Silica gel (Kieselgel 60 7749, PF_{254}, Merck, 50 g) was added to about 110 ml of cold distilled water. This slurry was shaken and was then quickly poured onto the circular glass plate before setting commences. The circular glass plate was then rotated while the gel was being poured to obtain an even setting. The plate was left to air-dry for about an hour before being dried in an oven at 80 ^{o}C for about 12 hours. The sample was dissolved in a minimum volume of a suitable solvent and loaded at the centre of the plate while the plate was spinning to form a thin band. Elution was then carried out with the appropriate solvent system. Fractions were collected, concentrated by rotary evaporator, examined by TLC and combined where appropriate.

Some of the solvent systems used as eluents were:

1. Chloroform
2. Chloroform : Hexanes
3. Chloroform with added 1% of concentrated ammonia
4. Chloroform : Methanol
5. Diethyl ether
6. Diethyl ether : Methanol
7. Diethyl ether with added 1% of concentrated ammonia
8. Ethyl acetate

9. Ethyl acetate : Hexanes
10. Ethyl acetate : Methanol
11. Ethyl acetate : with added 1% of concentrated ammonia

3.4 Spray Reagents (Dragendorff's Reagent)

Dragendorff's reagent was prepared as follow:

Solution A: 0.85 g of bismuth nitrate was dissolved in a mixture of 10 ml glacial acetic acid and 40 ml of distilled water.

Solution B: 8 g of potassium iodide was dissolved in 20 ml of distilled water

A stock solution was prepared by mixing equal volumes of solutions A and B. Dragendorff's reagent was made by mixing 1 ml of stock solution with 2 ml of glacial acetic acid and 10 ml of distilled water. Orange spots on the developed TLC plates indicated the presence of alkaloids.

3.5 Extraction of Alkaloids

The plant material was dried and ground and then extracted with distilled ethanol for two to three days. The ethanol extract was filtered and the residue was then re-extracted with a fresh portion of distilled ethanol. This procedure was repeated three times. The ethanol extract was concentrated to about 1/20 of its original volume under reduced pressure, before adding slowly to an excess of 5% hydrochloric acid with stirring. The acidic solution was then filtered through Kieselghur to remove the non-alkaloidal substances. The filtrate was then basified with concentrated ammonia solution to pH 10 and the liberated alkaloids were extracted exhaustively with chloroform. The chloroform extract was then washed with distilled water and dried over anhydrous

sodium sulphate. Finally, the solvent was removed under reduced pressure to furnish the crude alkaloidal mixture.

3.6 Isolation of Alkaloids

3.6.1 General Procedure

The crude alkaloidal mixture obtained from the extraction procedure described above was initially fractionated by flash column chromatography over silica gel. The column was eluted with chloroform, followed by a stepwise increase of methanol gradient. TLC was used to monitor the progress of the fractionation and also based on TLC, the many fractions collected were pooled into several major fractions, which were then subjected to further purification by flash column chromatography or preparative centrifugal TLC until pure compounds are obtained.

3.6.2 Isolation of alkaloids from the leaves of *Alstonia angustiloba* Miq.

Extraction of 21.0 kg of leaves material yielded 12.2 g of crude alkaloidal mixture. This mixture was then subjected to chromatography as summarized in the flow diagram shown in Figure 3.1, to yield the pure alkaloids.

3.6.3 Isolation of alkaloids from the stem-bark of *Alstonia angustiloba* Miq.

Extraction of 20.0 kg of stem-bark material yielded 18.0 g of crude alkaloidal mixture. This mixture was then subjected to chromatography as summarized in the flow diagram shown in Figure 3.2, to yield the pure alkaloids.

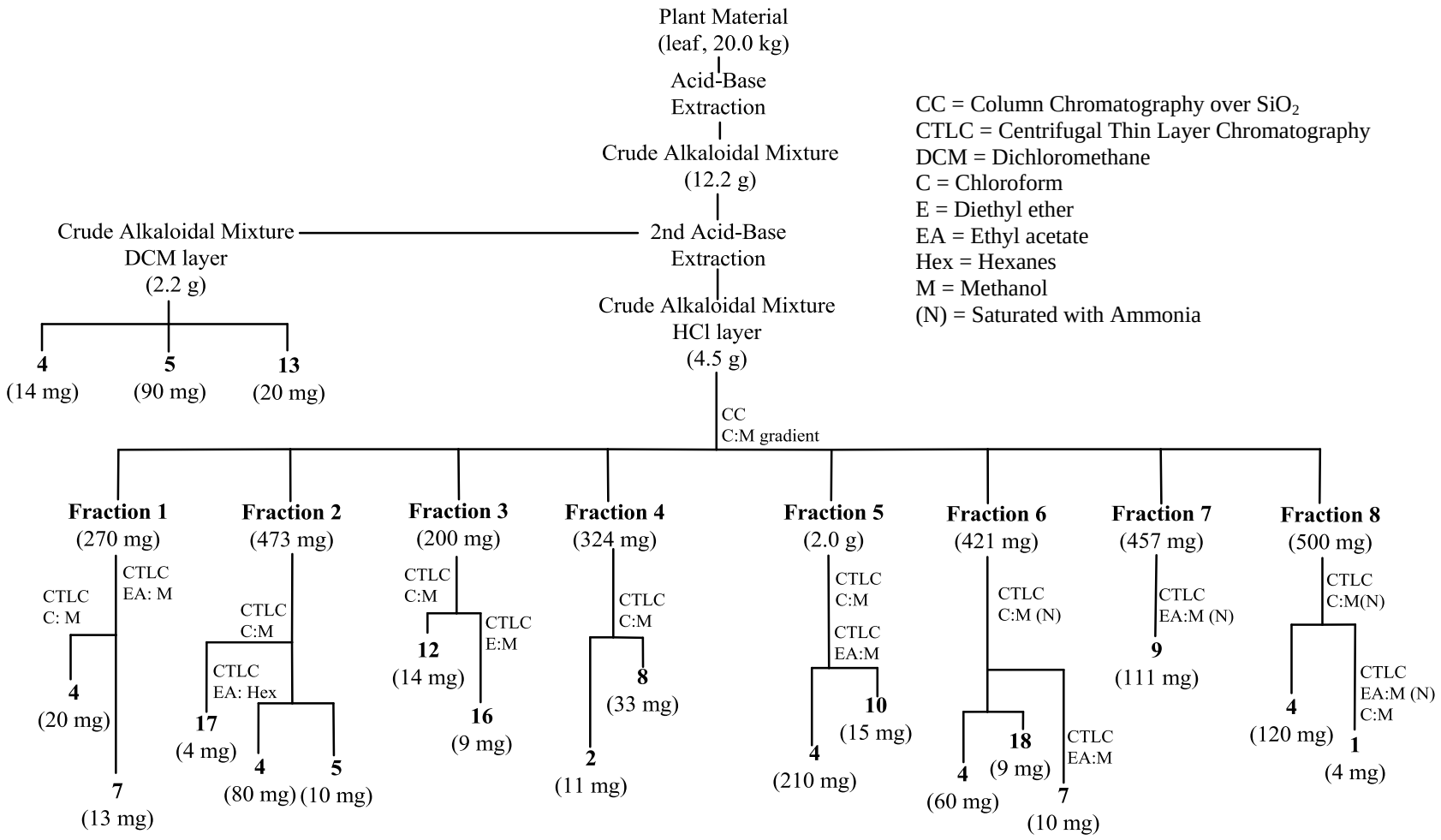
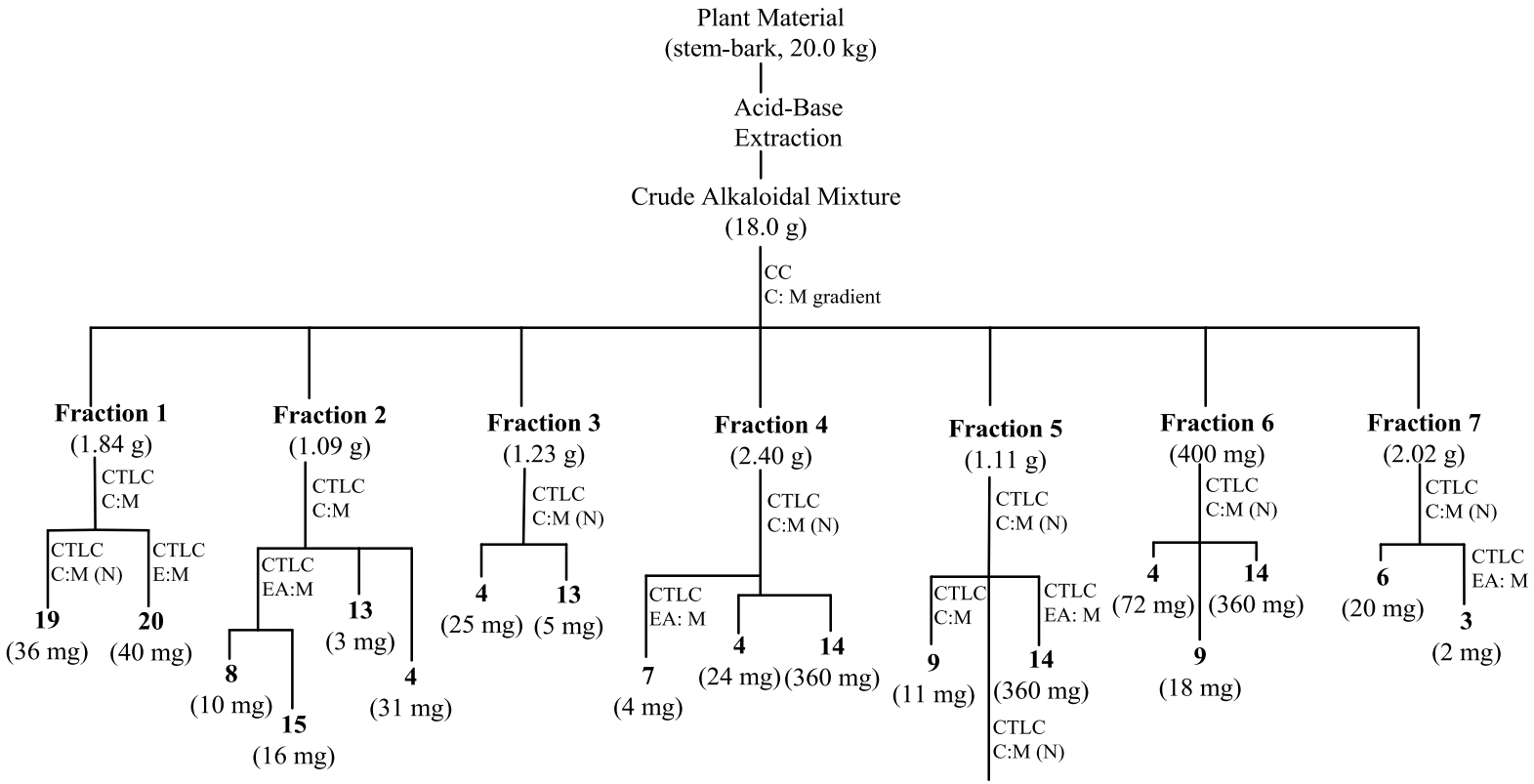


Figure 3.1. Isolation of alkaloids from the leaf extract of *Alstonia angustiloba* Miq.



CC = Column Chromatography over SiO_2
CTLC = Centrifugal Thin Layer Chromatography
C = Chloroform
E = Diethyl ether
EA = Ethyl acetate
Hex = Hexanes
M = Methanol
(N) = Saturated with Ammonia

Figure 3.2. Isolation of alkaloids from the stem-bark extract of *Alstonia angustiloba* Miq.

3.7 Compound Data

Angustilobine C (1): light yellowish oil; $[\alpha]^{25}_{D}$ −3 (*c* 0.13, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 222 (4.37), 283 (3.78) nm; IR (dry film) ν_{max} 3378, 1742, 1683 cm^{-1}; ESIMS *m/z* 373 $[MH]^+$; HRESIMS *m/z* 373.1760 (calc. for $C_{20}H_{24}N_2O_5$ + H, 373.1760); ^1H and ^{13}C NMR data, Table 2.2; HMBC: ^2J H(6) to C(7); H(15) to C(14), C(16); H(19) to C(18); NH to C(13); ^3J H(6), H(15), H(17) to C(2); H(6), H(15) to C(3); H(3) to C(6); H(9), NH to C(7); H(10), H(12), NH to C(8); H(11) to C(9); H(12) to C(10); H(9) to C(11); H(10) to C(12); H(9), H(11) to C(13); H(17) to C(15); H(14) to C(16); H(18) to C(17); H(17) to C(18); H(21) to C(19); H(6), H(18) to C(20); H(15), H(19) to C(21); H(17), $CO_2\underline{Me}$ to $\underline{CO_2}Me$. NOESY: H(6α)/H(3a); H(6β)/H(19); H(14b)/H(15); H(14a,b)/$CO_2\underline{Me}$; H(17β)/NH; H(21a)/H(3b), H(14b), H(15); H(21b)/H(15).

Andransinine (2): colourless block crystals (EtOAc); mp 212–214˚C; $[\alpha]^{25}_{D}$ −8 (*c* 0.13, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 223 (3.70), 284 (3.08) nm; IR (dry film) ν_{max} 3387, 2885, 2840, 1732 cm^{-1}; ESIMS *m/z* 381 $[MH]^+$; HRESIMS *m/z* 381.2178 (calc. for $C_{23}H_{28}N_2O_3$ + H, 381.2173); ^1H and ^{13}C NMR data, Table 2.3; HMBC: ^2J NH to C(2); H(6) to C(5); H(6) to C(7); H(9) to C(8); NH to C(13); H(22) to C(23); H(19) to C(18); H(21) to C(20); ^3J H(6), H(21) to C(2); H(5), H(15), H(21) to C(3); H(5), H(9), NH to C(7); H(6), H(10), H(12), NH to C(8); H(11) to C(9); H(12) to C(10); H(9) to C(11); H(10) to C(12); H(9), H(11) to C(13); H(19), H(22) to C(15); H(19) to C(17); H(15), H(21) to C(19); H(14), H(15) to C(20); H(5), H(19) to C(21); $CO_2\underline{Me}$, H(21) to $\underline{CO_2}Me$. NOESY: H(6α)/H(21); H(6β)/H(9); H(21α)/H(23); $CO_2\underline{Me}$/H(5α); $CO_2\underline{Me}$/H(15); $CO_2\underline{Me}$/H(19); NH/H(12).

Angustiphylline (3): light yellowish oil; $[\alpha]^{25}_{D}$ +16 (*c* 0.58, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 222 (3.70), 283 (3.08) nm; IR (dry film) ν_{max} 3387, 1732 cm^{-1}; ESIMS *m/z* 667 $[MH]^+$; HRESIMS *m/z* 667.3470 (calc. for $C_{39}H_{46}N_4O_6$ + H, 667.3490); ^1H and ^{13}C NMR data, Table 2.4; HMBC: ^2J H(21) to C(7); H(9) to C(8); NH to C(13); H(21) to C(20); H(6′) to C(7′); H(10′) to C(9′); NH′ to C(13′); H(19′) to C(18′); H(18′) to C(19′); H(15′), H(21′) to C(20′); ^3J H(17) to C(2); H(21) to C(3); NH, H(9) to C(7); H(10), H(12), H(21), NH to C(8); H(11) to C(9); H(12) to C(10); H(9) to C(11); H(10) to C(12); H(9), H(11) to C(13); H(20) to C(14); H(17), H(21) to C(15); H(20) to C(16); H(18) to C(17); H(21) to C(19); H(18) to C(20); H(6′) to C(21); H(15), H(17), CO_2Me to CO_2Me; H(6′), H(15′) to C(2′); H(15′) to C(3′); H(21) to C(6′); NH′, H(9′) to C(7′); H(6′), H(10′), H(12′), NH′ to C(8′); H(11′) to C(9′); H(12′) to C(10′); H(9′) to C(11′); H(10′) to C(12′); H(9′), H(11′) to C(13′); H(19′) to C(15′); H(15′) to C(17′); H(15′), H(21′) to C(19′); H(18′) to C(20′); H(15′), H(19′) to C(21′); H(15′), H(17′), CO_2Me′ to CO_2Me′; NOESY: H(3α)/H(14a); H(9)/H(21); H(14b)/H(15); H(15)/H(20); H(17a)/H(19b); H(17b)/H(18b); H(18a)/H(20); H(19a)/H(20); H(20)/H(3b); H(21)/H(3b); H(9′)/H(6′); H(14′b)/H(21′b); H(15′)/H(3a′); H(15′)/H(18′); H(21′a)/H(19′).

19,20-*E*-Vallesamine (4): light yellowish oil; $[\alpha]^{25}_{D}$ +14 (*c* 2.66, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 226 (4.15), 276 (3.88), 284 (3.92) 292 (3.84) nm; IR (dry film) ν_{max} 3348, 1726 cm^{-1}; ^1H and ^{13}C NMR data, Table 2.6 and 2.7, respectively; ESIMS *m/z* 341 $[MH]^+$, $C_{20}H_{24}N_2O_3$ + H.

17-*O*-Acetylvallesamine (5): light yellowish oil; $[\alpha]^{25}_{D}$ +371 (*c* 0.16, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 223 (4.55), 276 (3.90), 285 (3.92) 292 (3.86) nm; IR (dry film) ν_{max} 3390, 1733 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 2.6 and 2.7, respectively; ESIMS *m/z* 383 $[MH]^+$, $C_{22}H_{26}N_2O_4$ + H.

Yunnannesine (6): light yellowish oil; $[\alpha]^{25}_{D}$ +31 (*c* 3.16, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 222 (3.88), 280 (3.67), 283 (3.10), 295 (3.69) nm; IR (dry film) ν_{max} 3388, 1720 cm^{-1}; ^{1}H and ^{13}C NMR data, Tables 2.6 and 2.7, respectively; ESIMS *m/z* $[MH]^+$, $C_{19}H_{24}N_2O_3$ + H.

Angustilobine A (7): light yellowish oil; $[\alpha]^{25}_{D}$ +115 (*c* 0.02, MeOH); UV (EtOH), λ_{max} (log ε) 219 (4.08), 282 (3.54) nm; IR (dry film) ν_{max} 3350, 1726 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.8; ESIMS *m/z* 383 $[MH]^+$ $C_{22}H_{26}N_2O_4$ + H.

Angustilobine B (8): light yellowish oil; $[\alpha]^{25}_{D}$ −30 (*c* 0.44, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 223 (4.13), 285 (3.88) nm; IR (dry film) ν_{max} 3400, 1730 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.8; ESIMS *m/z* 339 $[MH]^+$ $C_{20}H_{22}N_2O_3$ + H.

***Nor*-6,7-*seco*angustilobine B (9):** light yellowish oil; $[\alpha]^{25}_{D}$ +166 (*c* 3.76, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 223 (4.34), 283 (3.86) nm; IR (dry film) ν_{max} 3380, 1725 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.9; ESIMS *m/z* 341 $[MH]^+$ $C_{20}H_{24}N_2O_3$ + H.

***Nor*-6,7-s*eco*-19,20α-epoxyangustilobine B (10):** light yellowish oil; $[\alpha]^{25}_{D}$ +55 (*c* 0.08, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 222 (3.90), 290 (3.88) nm; IR (dry film) ν_{max} 3360, 1740 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.9; ESIMS *m/z* 357 $[MH]^+$, $C_{20}H_{24}N_2O_4$ + H.

Undulifoline (11): light yellowish oil; $[\alpha]^{25}_{D}$ –33 (*c* 0.16, $CHCl_3$); UV (EtOH), λ_{max} (log ε) 220 (3.88), 288 (3.67) nm; IR (dry film) ν_{max} 3350, 1725 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.10; ESIMS *m/z* 341 $[MH]^+$ $C_{20}H_{24}N_2O_3$ + H.

Condylocarpine (12): light yellowish oil; $[\alpha]^{25}_{D}$ +501 (*c* 0.60, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 227 (4.03), 296 (3.84), 329 (3.95) nm; IR (dry film) ν_{max} 3363, 1673 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.11; ESIMS *m/z* 323 $[MH]^+$ $C_{20}H_{22}N_2O_2$ + H.

20*S*-Tubotaiwine (13): light yellowish oil; $[\alpha]^{25}_{D}$ +653 (*c* 0.10, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 210 (4.43), 231 (3.82), 295 (3.57) nm; IR (dry film) ν_{max} 3360, 1672 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.11; ESIMS *m/z* 325 $[MH]^+$ $C_{20}H_{24}N_2O_2$ + H.

***N*(4)-Demethylechitamine (14):** White amorphous; $[\alpha]^{25}_{D}$ +6 (*c* 1.00, $CHCl_3$); UV (EtOH), λ_{max} (log ε) 215 (4.36), 243 (3.83), 302 (3.47) nm; IR (dry film) ν_{max} 3350, 2975 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.12; ESIMS *m/z* 371 $[MH]^+$ $C_{21}H_{26}N_2O_4$ + H.

17-*O*-Acetyl-*N*(4)-demethylechitamine (15): light yellowish oil; $[\alpha]^{25}_{D}$ −17 (*c* 1.23, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 210 (4.36), 240 (3.83), 300 (3.47) nm; IR (dry film) ν_{max} 3350, 2977 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.12; ESIMS *m/z* 413 $[MH]^+$ $C_{23}H_{28}N_2O_5$ + H.

(–)-Alstolucine B (16): light yellowish oil; $[\alpha]^{25}_{D}$ −308 (*c* 0.29, $CHCl_3$); UV (EtOH) λ_{max} nm (log ε) 232 (3.85), 295 (3.75), 326 (3.91) nm; IR (dry film) ν_{max} 3361, 1704, 1678 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.13; ESIMS *m/z* 339 $[MH]^+$ $C_{20}H_{22}N_2O_3$ + H.

Vincamine (17): light yellowish oil; $[\alpha]^{25}_{D}$ +27 (*c* 0.14, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 226 (3.85), 279 (3.75) nm; IR (dry film) ν_{max} 1730, 742 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.14; ESIMS *m/z* 339 $[MH]^+$ $C_{20}H_{22}N_2O_3$ + H.

16*R*,19*E*-Isositsirikine (18): light yellowish oil; $[\alpha]^{25}_{D}$ −5 (*c* 0.23, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 227 (4.27), 282 (3.96), 290 (3.65) nm; IR (dry film) ν_{max} 3379, 2830, 2785, 1726, 1630 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.15; ESIMS *m/z* 355 $[MH]^+$ $C_{21}H_{26}N_2O_3$ + H.

Venoterpine (19): light yellowish oil; $[\alpha]^{25}_{D}$ −6 (*c* 0.58, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 262 (3.38) nm; IR (dry film) ν_{max} 3160, 1600 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.16; ESIMS *m/z* 150 $[MH]^+$ $C_9H_{11}NO$ + H.

Cantleyine (20): light yellowish oil; $[\alpha]^{25}_{D}$ −24 (*c* 1.02, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 260 (3.01) nm; IR (dry film) ν_{max} 3160,1700, 1630 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2.16; ESIMS *m/z* 208 $[MH]^+$ $C_{11}H_{13}NO_3$ + H.