Macroline, akuammiline, sarpagine, and ajmaline alkaloids from *Alstonia macrophylla*

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**A B S T R A C T**

A total of seventeen alkaloids, comprising six macroline (including alstofoline A, a macroline indole incorporating a butyrolactone ring-E), two ajmaline, one sarpagine, and eight akuammiline alkaloids, were isolated from the stem-bark and leaf extracts of the Malayan *Alstonia macrophylla*. The structure and relative configurations of these alkaloids were established using NMR, MS and in several instances, confirmed by X-ray diffraction analysis. Six of these alkaloids were effective in reversing multidrug-resistance (MDR) in vincristine-resistant KB cells.

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**Introduction**

Plants of the genus *Alstonia* (Apocynaceae), which are shrubs or trees, are distributed over tropical parts of Central America, Africa, and Asia (Whitmore, 1973; Markgraf, 1974; Sidiyasa, 1998) and are usually rich in alkaloids. A prominent feature of the *Alstonia* alkaloids is the preponderance of the macroline unit, which abounds in the alkaloids found in plants of the genus (Kam, 1999; Kam and Choo, 2006). About six species occur in Peninsular Malaysia and several of these (local name *Pulai*) are used in traditional medicine, for example, in the treatment of malaria and dysentery (Berkill, 1966; Perry and Metzger, 1980). In Peninsular Malaysia, these plants are mainly found in secondary and primary forest from sea level to about 3000 m altitude, as well as in swampy areas (Whitmore, 1973; Sidiyasa, 1998; Middleton, 2011). Recently the structure and absolute configurations of a number of new bisindoles isolated from a sample of *A. macrophylla* Wall collected from the western coast of Peninsular Malaya (Perak) were disclosed (Lim et al., 2011, 2012, 2013). In addition, a potentially useful method for the determination of the configuration at C-20 in E-seco macroline–macroline bisindoles, such as perhentinine, seco-macralstonine, and perhentidines A–C, was also reported. This was based on comparison of the NMR chemical shifts of the bisindoles and their acetate derivatives, in addition to X-ray determination of the absolute configuration of perhentinine and macralstonine (Lim et al., 2012). Reported herein are the isolation and structure determination of 17 new indole alkaloids (Fig. 1) from the leaf and stem-bark extracts of this plant.

**Results and discussion**

Compound 1 was a minor alkaloid isolated from the leaf extract of *A. macrophylla*. It was obtained as a light yellowish oil, with [α]D −104 (c 0.36, CHCl3). The UV spectrum showed two absorption bands (227 and 285 nm) characteristic of an indole chromophore. The IR spectrum showed a sharp band at 1769 cm−1 due to a lactone function. The EIMS of compound 1 had a molecular ion at m/z 296, and high resolution measurements yielded the molecular formula C18H18N2O2. Other notable fragment peaks observed at m/z 197, 182, 181, 170, and 144, are typical of macroline derivatives (Mayerl and Hesse, 1978), while the mass fragment at m/z 281 can be attributed to loss of a CH2. The 13C NMR spectrum (Table 1) displayed a total of 18 carbon resonances, corresponding to two methyl, three methylene, eight methine and five quaternary carbons. The presence of the lactone functionality, and an oxymethylene carbon, was supported by the observed carbon signals at δ 181.0 and δ 70.7, respectively. The 1H NMR spectrum (Table 2) showed the presence of an unsubstituted indole moiety (δ 7.11–7.49), two methyl groups corresponding to N1-Me at δ 3.64 and N4-Me at δ 2.42, and two downfield resonances at δ 4.42 (H-17β, t, J = 8 Hz) and 4.52 (H-17α, dd, J = 11, 8 Hz) due to the geminal...
hydrogens of an oxymethylene corresponding to C-17. The COSY and HSQC data disclosed partial structures that are characteristic of a macroline-type skeleton, such as NHCH₂ and NHCH₂-

28.4, 51.9, 3.61), a methyl ketone function (68.6, 1H and 41.8, 4.16 = 8 Hz) compared 29.1, 3.86, dd, J = 11 Hz) to the lactone carbonyl C-18 in the HMBC spectrum (Fig. 2). The presence of a lactone functionality as a part of ring E (a butyrolactone moiety) was deduced from the observed three-bond correlations from H-14 to both carbons (δ 126.5) and H-17α, to the lactone carbonyl C-18 in the HMBC spectrum (Fig. 2). The relative configurations at the various stereogenic centers of 1 was established by NOESY, and were similar to those in other macroline alkaloids. Alstofoline A (1) is the first example of a macroline indole alkaloid incorporating a γ-butyrolactone moiety in ring E.

Compound 3 was isolated in small amounts as a light yellowish oil, with [α]D 29.1 – 31 (c 0.11, CHCl₃). The IR spectrum showed a band at 1710 cm⁻¹ due to a ketone function. The presence of a ketone function was confirmed by the observed resonance at δ 208.6 in the ¹³C NMR spectrum. The ESIMS of 3 showed an [M+H]⁺ ion at m/z 339, which analyzed for C₁₂H₁₅NO₂. The UV spectrum showed absorption maxima at 228 and 286 nm, which are characteristic of an indole chromophore.

The ¹H and ¹³C NMR spectroscopic data (Tables 1 and 2) showed the presence of an unsubstituted indole moiety (δH 7.09–7.50, δC 108.7–120.9), two N-methyl signals (N4-Me, δC 41.8, δH 2.29; N1-Me, δC 29.1, δH 3.61), a methyl ketone function (δC 208.6; δH 28.4, δH 2.12), an oxymethylene, characteristic of C-17 in macroline alkaloids (δC 68.6, δH 3.72, and 3.95), and another oxymethylene signal at 64.3 (δH 3.86 and 4.18). The NMR signals, assigned with the aid of CO and HSQC, indicated that 3 is a macroline-type alkaloid. The NMR spectroscopic data resembled those of alstonerine (2), which was also isolated from the extract of this plant, except for the absence of signals associated with the trisubstituted C-20-C-21 double bond, such as the olefinic carbon resonances at C-20 (δ 126.5) and C-21 (δ 157.4), and the signal due to the vinylic H-21 in the ¹H NMR spectrum (δ 7.52). These resonances have in 3 been replaced by a methine at C-20 (δC 51.9, δH 1.97, m) and a methylene at C-21 (δC 64.3; δH 3.86, dd, J = 12.5, 3 Hz, δH 3.72, δH 3.18, d, J = 12.5 Hz), consistent with saturation of the C-20–C-21 double bond in 3. Less substantial changes were observed for the signals of carbons β to both carbons (C-20–C-21) in the ¹³C NMR spectrum. The configuration at C-20 can be deduced from the observed NOEs, viz., H-20/H-14β, H-18, H-21α; H-21β/H-14α, H-20, H-21β), which indicated the orientation of H-20α is α. Compound 3 is, therefore, the 20,21-dihydro derivative of alstonerine (2), which, while previously encountered as an intermediate compound in synthesis (Zhang and Cook, 1990), is here encountered as an optically active natural product for the first time.

Macropacrine D (4) was obtained as a light yellowish oil, with [α]D 0 – 43 (c 0.89, CHCl₃). It was isolated from the stem-bark extract of A. macrophylla, as well as A. angustifolia (Tan, 2011). The IR spectrum indicated the presence of hydroxyl and secondary amine...
functions at 3395 and 3292 cm$^{-1}$, respectively, while the UV spectrum indicated an indole chromophore ($\lambda_{max}$ 231 and 286 nm). The ESIMS of 4 showed an [M+H]$^+$ peak at $m/z$ 439, and high-resolution measurements yielded the molecular formula C$_{26}$H$_{34}$N$_2$O$_2$. The $^{13}$C NMR spectrum (Table 1) had a total of 26 carbon signals comprising three methyl, seven methylene, ten methine and six quaternary carbons, in agreement with the molecular formula. The observed carbon resonance at $\delta$ 70.1 was due to an oxymethine carbon, while the carbon resonances at $\delta$ 64.6 (C-17) and 64.0 (C-26) were due to two oxymethylenes at $\delta$ 3.62, 2.33, and 1.62, respectively, and two sets of resonances due to two oxymethylene carbons. In addition, two low-field quaternary carbon signals were observed at $\delta$ 108.6 and 105.1, each of which was attached to two oxygen atoms. The $^1$H NMR spectrum (Table 2) showed the presence of an unsubstituted indole moiety (at $\delta$ 7.11–7.13, 7.04–7.06, and 6.97–6.99), three methyl groups corresponding to N1-Me, N4-Me, and 18-Me, at $\delta$ 3.62, 2.33, and 1.62, respectively, and two sets of resonances due to two oxymethylene protons at $\delta$ 3.75, 4.05, and at $\delta$ 3.39, 3.84.

The COSY spectrum disclosed some partial structures, which are characteristic of a macroline-type skeleton, such as NCHCH$_2$O, CHCH$_2$ and NCH$_2$CHCH$_2$O, corresponding to the N4-C5-C6, C20–C21, and N4-C3–C14–C15–C16–C17–O fragments,
respectively. An additional fragment, viz., OCH\textsubscript{2}CH\textsubscript{2}O, was also indicated from the COSY spectrum which, taken with the other NMR spectroscopic data, indicated affinity to the macrodasine group of alkaloids reported recently (Tan et al., 2011). These macroline alkaloids incorporate additional fused spirocyclic tetrahydrofuran–tetrahydrofuran (macrodasines A (8), B, C, G) and tetrahydrofuran–tetrahydropyran (macrodasines D, E, F (7)) rings. The OCH\textsubscript{2}CH\textsubscript{2}O partial structure noted from the COSY spectrum which, taken with the other correlations observed in the HMBC spectrum, viz., H-26b, H-24 to C-22, H-24 to C-26, H-25 to C-23, and, H-21 to C-15, C-23. The proposed structure is consistent with the full HMBC data (Fig. 3). Compound 6 differs from macrodasine F (7), by the absence of the OH substituent at C-25.

The ring junction stereochemistries between rings C, D, E, and F were deduced to be similar to those of macroline alkaloids, as well as macrodasines (A–G) from the NOE data. The reciprocal NOEs observed for H-23/H-21 and H-24a, H-25a, and H-26a suggested a chair conformation adopted by the tetrahydropyran ring G, as shown in Fig. 3, in which H-24a, H-25a, and H-26a are all axially oriented. As in the case of macrodasine F (7), a NOE was not observed between the C-26 hydrogens and 18-Me, suggesting that the configuration of the spirocyclic C-22 is S, i.e., the C-26 hydrogens and 18-Me are directed away from each other (Tan et al., 2011).

Alstonoxine C (9) was isolated as a light yellowish oil, with [\alpha]\textsubscript{D} \textsuperscript{0} +30 (c 0.39, CHCl\textsubscript{3}), and subsequently crystallized from CH\textsubscript{2}Cl\textsubscript{2}–hexane as colorless block crystals. The UV spectrum showed absorption maxima at 216, 266, and 291 nm, indicative of an oxin-
doxide chromophore. The IR spectrum showed the presence of lactam and ketone carbonyl (1694 cm\(^{-1}\)), NH (3295 cm\(^{-1}\)), and hydroxyl (3390 cm\(^{-1}\)) functions, while the carbon resonances at \(\delta\) 183.0, 208.7, 65.9 confirmed the presence of lactam (oxindole), ketone, and oxymethylene groups, respectively. The ESIMS showed an [M+H]\(^+\) peak at \(m/z\) 359, analyzing for \(C_{20}H_{28}N_2O_4\). The \(^1\)H NMR spectroscopic data (Table 2) showed features typical of macroline oxindole alkaloids. Notable features include presence of an N1-Me (\(\delta\) 3.16 s), a methyl ketone (\(\delta\) 3.80 m, and 4.01 br d, \(J = 18\) Hz), an oxymethylene (\(\delta\) 3.80 m, and 4.01 br d, \(J = 12\) Hz; \(\delta\) 65.9; C-17). In addition, signals due to the presence of a methylene group to a carbonyl group were observed at \(\delta\) 2.48 (dd, \(J = 18, 5\) Hz) and 2.80 (dd, \(J = 18, 8\) Hz). These features are reminiscent of the C-18–C-19–C-20 side-chain in the E-seco oxindole alstonoxine A (10). The NMR spectroscopic data (Tables 1 and 2) of 9 did in fact show a close resemblance to those of alstonoxine A (10) (Kam and Choo, 2000), except for the absence of an aromatic hydrogen resonance at C-11, which was replaced in 9 by a 3H singlet at \(\delta\) 3.84 due to an aromatic methoxy substituent. The placement of the latter at C-11 was further confirmed by the observed NOEs between OMe and H-10, as well as H-12. The configuration of the spirocyclic C-7 was assigned as S from the observed reciprocal NOEs between H-9 and H-15. In view of the availability of suitable crystals (crystals were obtained from CH\(_2\)Cl\(_2\)–hexane), an X-ray diffraction analysis was carried out which provided confirmation of the structure and relative configuration of alstonoxine C (9) deduced from the spectroscopic data (Fig. 4). The X-ray structure of alstonoxine C (9) also provided additional support for the original assignment of the structure and relative configuration of alstonoxine A (10).

Alstonoxine D (11) was isolated as a light yellowish oil, with \([\alpha]_D -16\) (c 0.23, CHCl\(_3\)). The UV and IR spectra of 11 were similar to those of alstonoxine B (12) (Kam and Choo, 2000), while the ESIMS showed an [M+H]\(^+\) peak at \(m/z\) 361 (\(C_{20}H_{28}N_2O_4 + H\)). The \(^1\)H and \(^13\)C NMR spectroscopic data (Tables 1 and 2) of 11 were generally similar to those of alstonoxine B (12), except for the presence of an additional aromatic methoxy singlet at \(\delta\) 3.83, and the absence of one aromatic-H signal. Placement of the methoxy substituent at C-11 was supported by the observed coupling behaviour of the aromatic hydrogens, as well as from the observed three-bond correlations from H-9 to C-11 and C-13, in the HMBC spectrum. This assignment was further supported by the observed NOEs between 11-OMe and H-10, H-12. The configuration of the spirocenter C-7 was assigned as S from the observed NOEs between H-9 and H-15. The difference between alstonoxine D (11) and the previous compound 9 resides in the C-18–C-19–C-20 side-chain. Whereas C-19 in 9 is a ketone carbonyl, the C-19 in 11 is an oxymethine associated with a secondary alcohol functionality. This difference was also reflected in the respective NMR spectroscopic data of 9 and 11. The NMR data of 11 were, however, insufficient to establish the stereochemistry of C-19. Towards this end, alstonoxine C (9) was treated with NaBH\(_4\)/MeOH, which gave a mixture of the epimeric alcohol products in approximately equal amounts, which were separated by preparative centrifugal TLC (SiO\(_2\), 1% MeOH/CHCl\(_3\), NH\(_3\)-saturated). The NMR data indicated that the slower eluting compound corresponded to compound 11. With sufficient amounts obtained in this manner, the configuration at C-19 could be determined by Horeau’s procedure (see Experimental) (Horeau and Kagan, 1964; Barnekow and Cardellina, 1989), which showed that the C-19 configuration in 11 is S (the faster eluting compound 11a therefore corresponded to the 19R epimer). The structure and relative configuration of alstonoxine B (12) was previously reported from Alstonia angustifolia var. latifolia (Kam and Choo, 2000), based on analysis of the NMR and MS data which at the time were insufficient to assign the configuration at C-19. Alstonoxine B (12) was also isolated in the present study, and since suitable crystals were obtained from CH\(_2\)Cl\(_2\)–hexane.
solution in this instance, X-ray diffraction analysis was carried which established the relative configuration of C-19 in alstonoxine B (12) as S (Fig. 5). The X-ray structure of 12 also provided additional support for the determination of the C-19 configuration of 11 as 19S, using Horeau’s procedure (vide supra), since the NMR spectroscopic data of 11 and 12 indicated that the non-aromatic portion of 11 was virtually identical to the non-aromatic portion of 12. Therefore the configuration of C-19 in 11 can be assumed to be similar to that of alstonoxine B (12).

Compound 13 was obtained as a light yellowish oil, [α]D +8° (c 0.24, CHCl3). The UV spectrum showed absorption maxima at 229, 254, and 284 nm, indicative of an indole chromophore. The IR spectrum had a broad band at 3372 cm⁻¹ due to an OH function. The ESIMS of 13 showed an [M+H]⁺ peak at m/z 309, which analyzed for C₁₀H₁₈N₂O. The ¹³C NMR spectrum (Table 1) displayed a total of 20 resonances, comprising two methyl, four methylene, nine methine, and five quaternary carbon atoms, in agreement with the molecular formula. Analysis of the ¹H NMR data (Table 2) established the presence of an unsubstituted indole moiety (δ 7.08–7.41), an N1-Me (δ 3.63), an oxymethylene associated with a hydroxymethyl group (δ 3.47), and an ethylenyl side-chain (δ 1.57, 5.28). The COSY spectrum yielded a fragment consistent with a sarpagine-type compound, viz. NCH₂CH₂CH₂(OH)CH₂CH₂. Analysis of the 2-D NMR spectroscopic data indicated that 13 possessed the same molecular connectivity as affinisine (14) (Clivio et al., 1991), which was also isolated. The ¹H and ¹³C NMR spectroscopic data (Tables 1 and 2) of 13 were generally similar to those of 14 except for notable differences in the chemical shifts of C-15 in the ¹³C NMR spectrum, and H-15 in the ¹H NMR spectrum. The configurations at the various stereogenic centers were deduced from the NOESY data which indicated similarity with those in affinisine. The observed H-16/H-6β NOE indicated that the configuration of C-16 is R. This left the geometry of the C-19–C-20 double bond as a possible point of departure between the two compounds. This was confirmed by the observed reciprocal NOEs for H-19/H-15 and H-21/H-18, which established the geometry of the 19,20-double bond as Z. Compound 13 is therefore the 19,20-Z isomer of affinisine (19,20-E).

Compound 15 (2(R)-3-hydroxycathafoline) was isolated as a light yellowish oil, with [α]D +48° (c 0.24, CHCl3). The IR spectrum showed a band at 1739 cm⁻¹ due to an ester group. The UV spectrum showed typical dihydroindole absorptions at 202, 252, and 295 nm. The ESIMS of 15 showed an [M+H]⁺ peak at m/z 355, which analyzed for C₁₂H₁₈N₂O₂. The ¹³C NMR spectrum (Table 1) displayed a total of 21 resonances, comprising three methyl, four methylene, eight methine, and six quaternary carbon atoms. The ¹H NMR spectrum (Table 2) showed the presence of an unsubstituted indole moiety (δ 6.61–7.09), an N1-Me (δ 2.95), a methyl ester (δ 3.78), an aminomethylene (δ 2.98, 4.10; δc 55.0), and an ethylenyl side-chain (δ 1.49, 5.41). The COSY spectrum yielded the following fragments, viz., NCH₂CH₂ and CH₂CHCH. The ¹³C NMR spectrum (Table 1) showed the presence of two downfield resonances, a methine at δ 80.7, and an oxygenated quaternary carbon at δ 85.3. The former resonance was characteristic of C-2 of cathafoline alkaloids with a H-2β orientation (Das et al., 1977), while the latter resonance at δ 85.3 was characteristic of a carboline moiety, suggesting the presence of hydroxy-substitution at C-3. This was further supported by the observed three bond correlations of 15 to H-3 and H-21 to the oxygenated C-3 in the HMBC spectrum (Fig. 6). The ¹H and ¹³C NMR spectroscopic data of 15 showed a close correspondence with those of cathafoline (18) (Alta-ur-Rahman et al., 1988), except for the downfield shift of the C-3 signal in the ¹³C NMR spectrum (from δ 47.2 in 18 to δ 85.3 in 15) due to substitution by the OH group. The configurations at the various stereogenic centers of 15 were similar to those of cathafoline (18) as indicated by the observed NOEs from the NOESY spectrum (Fig. 6). The observed NOE between H-16 and H-9 indicated that the configuration of C-16 is R (H-16 directed towards the indole moiety). The observed NOE between H-16 (δ 2.90) and the H-14 signal at δ 1.90 allowed the attribution of this signal to H-14β. The NOE between this H-14β and H-2 indicated that the or-
entation of H-2 is β as in cathafoline (2R), which was also consistent with the observed C-2 resonance at δ 79.1. Furthermore, in the cathafoline alkaloids, the rigid architecture of the molecule restricts the orientation of any substituent at C-3 (including H) to be α only and as such, the orientation of the C-3—OH is therefore by necessity, α. This is the first isolation of a naturally occurring 3-hydroxycathafoline, although a compound previously assigned as 3-hydroxycathafoline has been encountered in transformations of the echitamine alkaloids (Massiot et al., 1983; the NMR spectroscopic data for compounds 17–22, 24, 26, and 28).

Table 3
$^1$H (400 MHz) NMR spectroscopic data for compounds 17, 19–22, 24, 26, and 28.a

<table>
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<tr>
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<th>17</th>
<th>19</th>
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<td>21a</td>
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<td>3.00 br d (15)</td>
<td>3.91 br d (15)</td>
<td>3.02 br d (15)</td>
<td>3.85 br d (15)</td>
<td>3.06 br d (18)</td>
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<td>21b</td>
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<td>3.81 m</td>
<td>4.20 m</td>
<td>3.84 m</td>
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<td>2.63 s</td>
<td>3.07 s</td>
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<td>CO2Me</td>
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<td>5′</td>
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<td>–</td>
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<tr>
<td>H26</td>
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</tr>
<tr>
<td>H22</td>
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<td>–</td>
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</tr>
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</table>

a Measured in CDCl3.
b Assignments may be reversed.

The 13C NMR spectrum (Table 1) showed a total of 21 resonances, comprising three methyl, four methylene, nine methine, and five quaternary carbon atoms. The 1H NMR spectrum (Table 2) showed many features which were also present in 15 indicating the presence of similar groups, such as an unsubstituted indole moiety, an N1-Me, a methyl ester, an aminomethylene, and an ethylenide side-chain. The COSY spectrum, however, showed the presence of the following partial structures, viz., NCH2CH2 and CHCH2CH2CH2, which differed from the previous compound by the replacement of CH2CHCH with a CHCH2CH2CH2 fragment. Furthermore, the downfield resonance, at δ 85 in the 13C NMR spectrum, associated with the carbinolamine moiety in 15, was not observed in the spectrum of 16. Comparison of the 1H and 13C NMR spectroscopic data (Tables 1 and 2) indicated a general similarity with those of cathafoline (18), except for the more notable differences in the chemical shifts of C-2 and C-14 in the 13C NMR spectrum, and H-2 in the 1H NMR spectrum. This observation, as well as the fact that 16 is isomeric with cathafoline (18) as shown by the MS data (vide supra), suggested that 16 is a stereoisomer of 18. The relative configurations at the various stereogenic centers of 16 were similar to those of cathafoline (18) as deduced from the NOESY data, except for the configuration at C-2. In the case of 16, a NOE was not observed between H-2 and H-14β (which was the case in 15), but was instead observed between H-2 and H-6x, N1-Me, and H-3. These NOEs were different from those observed in the 2R cathafoline alkaloids, and indicated that the relative configuration of C-2 in 16 is S (H-2α) (Fig. 7), i.e. an inference which was also consistent with the observed resonance of C-2 at δ 70.1 (Das et al., 1977; Massiot et al., 1983). Since suitable crystalline 16 were obtained from CH2Cl2–hexane, an X-ray diffraction analysis was carried out which confirmed the assignment of configuration of C-2 as S based on the NMR spectroscopic data (Fig. 8). Compound 16 is therefore the C-2 epimer of cathafoline (18).

Compound 17 (2S)-10-methoxycathafoline was obtained as a light yellowish oil, with $\delta_{13} 137.5$ (c 0.27, CHCl3). The UV and IR spectra were similar to those of compound 16. The ESIMS showed
an [M+H]+ peak at m/z 369, which is 30 mass-units more than 16. The NMR spectroscopic data of 16 and 17 (Tables 1 and 3) were generally similar, except for the presence of a methoxy group in 17. The substitution of the methoxy group at C-10 was deduced from the coupling behaviour of the aromatic hydrogens, the observed three-bond correlations from H-12 and 10-OMe to C-10 in the HMBC spectrum, and the following observed NOEs: H-9/10-OMe; H-11/10-OMe; H-12; and, H-12/H-11, N1-Me. In view of the diagnostic NOEs observed for H-2/H-6z, N1-Me, H-3, and the observed C-2 resonance at δ 70.7, the configuration at C-2 in 17 is therefore assigned as 2(S)-10-methoxy cathafoline.

Compound 19 (10-demethoxy vincorine) was isolated as a light yellowish oil, with [α]D −86 (c 0.48, CHCl3). The UV spectrum showed dihydroindole absorption maxima at 208, 256 and 317 nm, while the IR spectrum indicated the presence of an ester carbonyl (1734 cm−1) function. The ESIMS of 19 gave an [M+H]+ peak at m/z 399, corresponding to the molecular formula C21H23O4N2, differing from vincorine (23) by addition of 30 mass units. The 1H and 13C NMR spectroscopic data (Tables 1 and 3) were similar in all respects to those of 23, except for the aromatic resonances and the presence of an additional aromatic methoxy substituent at δH 3.86 (6:56:4). In the 1H NMR spectrum of 21, two aromatic singlets were observed at δ 7.02 and 5.96, which were assigned to H-9 and H-12, respectively based on the NOE data (NOEs observed for H-9/10-OMe, H-12/NMe, 11-OMe). These features are consistent with 10,11-dimethoxy-substitution on the indole moiety. Compound 21 is therefore assigned as 11-methoxy vincorine.

Compound 22 (vincorine N(4)-oxide) was obtained as a yellowish oil, with [α]D −84 (c 0.4, CHCl3). The UV spectrum showed dihydroindole absorption maxima at 211, 250 and 323 nm) was characteristic of a dihydroindole chromophore, while the IR spectrum showed the presence of an ester carbonyl (1734 cm−1) function. The ESIMS of 22 had an [M+H]+ peak at m/z 385, which analyzed for C21H22O4N2O, 16 mass units higher than that of 19. Compound 20 was readily identified as the N4-oxide of 10-demethoxy vincorine (Table 3), in particular, the characteristic downfield shifts of the carbon resonances for C-2, C-5, and C-21, when compared with those of 10-demethoxy vincorine (19).

Compound 20 (10-demethoxy vincorine N(4)-oxide) was obtained as a light yellowish oil, with [α]D −62 (c 0.5, CHCl3). The UV spectrum (208, 247, and 299 nm) showed absorption maxima characteristic of a dihydroindole chromophore, while the IR spectrum showed the presence of an ester carbonyl (1734 cm−1) function. The ESIMS of 20 showed an [M+H]+ peak at m/z 355, which analyzed for C21H22O4N2O5, 16 mass units higher than that of 19. Compound 20 was readily identified as the N4-oxide of 10-demethoxy vincorine from its NMR spectroscopic data (Tables 1 and 3), in particular, the characteristic downfield shifts of the carbon resonances for C-2, C-5, and C-21, when compared with those of 10-demethoxy vincorine (19).

### Table 4

<table>
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<th>Compound</th>
<th>IC50 µg/ml KB/S</th>
<th>IC50 µg/ml KB/VJ300</th>
<th>IC50 µg/ml KB/VJ300 (+)</th>
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</thead>
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<tr>
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<td>Macrocarpine D (4)</td>
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<td>Macrodon H (6)</td>
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</table>

*a* KB/S and KB/VJ300 are vincristine-sensitive and vincristine-resistant human oral epidermoid carcinoma cell lines, respectively.

*b* With added vincristine, 0.1 µg/ml, which did not affect the growth of the KB/VJ300 cell.

*c* With added vincristine, 0.25 µg/ml, which did not affect the growth of the KB/VJ300 cell (Nam et al., 1998b).
from its NMR spectroscopic data (Tables 1 and 3), in particular the characteristic downfield shifts of the carbon resonances for C-2, C-5, and C-21, when compared with those of vincorine (23).

Compound 24 (11-demethoxyquaternine) was obtained as a light yellowish oil, with δ 6.79 (3H, s, CH3O). The UV spectrum had absorption maxima at 208, 241, 307 nm, suggesting the presence of a dihydroindole chromophore, and the IR spectrum displayed an ester carbonyl band at 1736 cm⁻¹ (δc 172.5). The ESIMS showed an [M+H]+ peak at m/z 383, which analyzed for C22H22N2O4. The 1H and 13C NMR spectroscopic data (Tables 1 and 3) established the presence of a substituted indole moiety, an ethylidene side-chain, and three 3H singlets at δH 3.70, 3.64, and 2.90, due to a methyl ester (δc 172.5, 51.7), an aromatic methoxy (δc 56.0), and an NMe (δc 30.0) group, respectively. In addition, a deshielded methine was observed as a doublet at δH 4.70 (J = 3 Hz), with the corresponding carbon resonance observed at δc 87.2. The observed downfield 1H and 13C shifts shown by this methine, is characteristic of a carbon, which is adjacent to a nitrogen and an oxygen atom. The NMR spectroscopic data indicated a similarity to those of quaternine (25) (Abbe et al., 1994a), which was also present in the same plant. The main difference noted from the NMR spectroscopic data was the absence of one of the aromatic methoxy groups (two aromatic methoxy groups were present in quaternine). The aromatic doublet at δ 6.76 was assigned to H-9 from its NOE with H-6α (Fig. 10), while the other aromatic doublet at δ 5.54 was assigned to H-12 from the observed NOE between these hydrogen and N1-Me. The observed NOEs for OMe/H-9, H-11 and H-14 confirmed the placement of the aromatic methoxy group at C-10.

The relative configuration of C-16 was assigned as R’ (H-directed towards the indole moiety), from the observed NOEs between H-16 and H-14(α), H-15 (Fig. 10).

Compound 26 (vincamajine N(4)-oxide) was obtained as a yellowish oil, with δH δD –29 (c 0.04, CHCl3). The UV spectrum (210, 231 and 282 nm) was characteristic of a dihydroindole chromophore, while the IR spectrum showed the presence of OH and ester groups (3400 and 1737 cm⁻¹). The presence of an ester function was further confirmed by the observed signals at δ 52.3 and 170.8 in the 13C NMR spectrum. The ESIMS of 26 showed an [M]+ peak at m/z 383 (C22H22N2O4), which was 16 mass units higher than that of vincamajine (27). The NMR spectroscopic data (Table 1 and 3) indicated an alkaloid of the ajmaline type and were in fact similar to those of vincamajine (27) (Cherif et al., 1989), which was also isolated, except that the resonances of C-3, C-5, and C-21, were shifted downfield in 26. Based on these observations, compound 26 was readily identified as the N4-oxide of vincamajine.

Compound 28 (vincamajine 17-0-veratrate N(4)-oxide) was obtained as a yellowish oil, with δH δD –75 (c 0.94, CHCl3). The UV spectrum (209, 254 and 292 nm) was characteristic of a dihydroindole chromophore, while the IR spectrum showed the presence of an ester (1737 cm⁻¹) function. The ESIMS of 28 had an [M]+ peak at m/z 547, which analyzed for C31H35N2O5. The 1H NMR (Table 3) spectrum showed similar features as those shown by vincamajine (27), except for the presence of additional signals due to the acid residue (3’,4’-dimethoxybenzoic acid or veratic acid) associated with an ester group at C-17 (vincamajine 17-0-veratrate (29)) (Abbe et al., 1994b), and the downfield shifts of the carbon resonances for C-3, C-5, and C-21, when compared with those of vincamajine 17-0-veratrate (29). Compound 28 (measured mass was 16 mass units higher than that of 29) is therefore readily identified as the N4-oxide of vincamajine 17-0-veratrate.

All the new compounds tested showed no appreciable cytotoxicity against drug-sensitive and vincristine-resistant KB cells (K50 > 25 μg/ml in all cases). However, 11-methoxyvincorine (21), 11-demethoxyquaternine (24), 19,20-Z-affinisine (13), 2(S)-10-methoxycafarofoline (17), 10-demethoxyvincorine (19), and macrodasine H (6), were found to reverse multidrug resistance in vincristine-resistant KB (VJ300) cells (Table 4).

The structures of several new macroline oxindole alkaloids from various Alstonia species were previously reported (Kam and Choo, 2000). In view of a systematic error involving the labeling of the configuration (R or S) at the spirocyclic carbon (C-7) in these alkaloids, a list of these alkaloids is now included with the correct R/S labeling for C-7: isomalstonine (reported 7S, corrected 7R) (Kam and Choo, 2000), macrogentine (reported 7S, corrected 7R) (Kam and Choo, 2000), alstonoxine A (reported 7R, corrected 7S) (Kam and Choo, 2000), alstonoxine B (reported 7R, corrected 7S) (Kam and Choo, 2000), alstofoline (reported 7R, corrected 7S) (Kam and Choo, 2000), N1-demethylalstonisine (reported 7R, corrected 7S) (Kam and Choo, 2000), affinisine oxindole (reported 7R, corrected 7S) (Kam and Choo, 2004b).

Concluding remarks

The present investigation reports the presence of 17 new indole alkaloids and completes the study of the alkaloids of this particular sample of A. macrophylla. The new indole alkaloids found were mainly of the macroline, sarpagine, akamullamine/vincorine and ajmaline types. It is noted that there is some variation in the alkaloid composition between the indole alkaloids found in the present sample collected on the western coast of Peninsular Malaysia (Perak), compared to a sample collected from the eastern coast of Peninsular Malaysia (Terengganu) (Kam and Choo, 2004a). Although a number of alkaloids from the various subtypes were common in both samples, nevertheless, alkaloids which were found in one sample, were absent in the other, and vice versa. For instance, sarpagine and ajmaline alkaloids found in the present sample were not found in the previous sample. There is also a greater predominance of the akamullamine alkaloids, as well as bisindole alkaloids (Lim et al., 2011, 2012, 2013) in the present sample. Among the alkaloids tested, the akamullamine alkaloids, 11-methoxyvincorine (21) and 11-demethoxyquaternine (24), were the most active in reversing multi-drug resistance in vincristine-resistant KB cells.

Experimental

General

Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO P-1020 digital polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum 400 spectrophotometer. UV spectra were obtained on a Shimadzu UV-3101PC spectrophotometer. 1H and 13C NMR spectra were recorded in CDC13 using TMS as internal standard on JEOL JNM-LA 400 and JNM-ECA 400 spectrometers at 400 and 100 MHz, respectively. NOESY experiments were carried out on a Bruker Avance III 600 spectrometer at 600 MHz. ESIMS and HRESIMS were obtained on an Agilent 6530 Q-TOF or on a JEOL AccuTOF-DART mass spectrometer.

Plant material

A. macrophylla was collected in Perak, Malaysia and identification was confirmed by Dr. Richard C. K. Chung, Forest Research Institute, Malaysia, and Dr. K. T. Yong, Institute of Biological Sciences, University of Malaya. Herbarium voucher specimens (K671) are deposited at the Herbarium, University of Malaya.
The leaf (7 kg) and stem-bark (9 kg) were exhaustively extracted with EtOH at room temperature and the concentrated EtOH extract was then partitioned with dilute acid (3% tartaric acid) followed by basification of the aqueous fraction with concentrated NH₄OH solution and extraction of the liberated alkaloids with CHCl₃. The alkaloids were isolated by initial silica gel column chromatography (CC) using CHCl₃ with increasing proportions of MeOH, followed by further chromatography of the appropriate partially resolved fractions using centrifugal preparative TLC. The solvent systems used for centrifugal preparative TLC were Et₂O–hexane (4:1; NH₄+-saturated), Et₂O–EtOH (NH₄+-saturated), Et₂O–MeOH–NH₃ (100:1; NH₄+-saturated), Et₂O–MeOH–NH₃ (20:1; NH₄+-saturated), Et₂O–MeOH–NH₃ (10:1; NH₄+-saturated), EtOAc–hexane (1:5; NH₄+-saturated), EtOAc–hexane (1:4; NH₂-saturated), EtOAc–hexane (1:1; NH₄+-saturated), EtOAc–hexane (2:1; NH₂-saturated), EtOAc–hexane (3:1; NH₂-saturated), EtOAc–MeOH (NH₄-saturated), EtOAc–MeOH (10:1; NH₂-saturated), EtOAc–MeOH (10:1; NH₂-saturated), EtOAc–MeOH (10:1; NH₂-saturated), EtOAc–MeOH (10:1; NH₂-saturated), EtOAc–MeOH (10:1; NH₂-saturated), EtOAc–MeOH (10:1; NH₂-saturated). The yields (mg kg⁻¹) of the alkaloids from the stem-bark extract were as follows: 1 (0.9), 9 (7.9), 13 (4.0), 15 (5.0), 16 (4.7), 17 (6.9), 19 (1.0), 20 (1.8), 21 (0.7), 22 (0.6), 24 (13.2), 26 (0.2), and 28 (1.9). The yields (mg kg⁻¹) of the alkaloids from the leaf extract were as follows: 3 (0.2), 4 (3.5), 6 (0.3), 11 (2.2), 13 (4.3), and 16 (0.1).
Vincorine N(4)-oxide (22)

Light yellowish oil; [α]D25 −84 (c 0.4, CHCl3); UV (EtOH) λmax (log ε) 211 (4.88), 250 (4.51), 323 (4.05) nm; IR (dry film) νmax 1734 cm−1; for 1H NMR and 13C NMR spectroscopic data, see Tables 3 and 1, respectively; ESIMS m/z: 385 [M+H]+; HRESIMS m/z: 385.2130 [M+H]+ (calcd for C21H22N2O3 385.1971). Vincamine N(4)-oxide (26)

Light yellow oil; [α]D25 −10 (c 0.21, CHCl3); UV (EtOH) λmax (log ε) 208 (4.64), 241 (4.35), 307 (3.96) nm; IR (dry film) νmax 1736 cm−1; for 1H NMR and 13C NMR spectroscopic data, see Tables 3 and 1, respectively; ESIMS m/z: 383 [M+H]+; HRESIMS m/z: 383.1978 [M+H]+ (calcd for C22H22N2O3 383.1980).

Vincamajine N(4)-oxide (28)

Light yellow oil; [α]D25 −29 (c 0.04, CHCl3); UV (EtOH) λmax (log ε) 210 (4.94), 231 (4.70) and 282 (4.30) nm; IR (dry film) νmax 3400 and 1737 cm−1; for 1H NMR and 13C NMR spectroscopic data, see Tables 3 and 1, respectively; ESIMS m/z: 383 [M+H]+; HRESIMS m/z: 383.1980 [M+H]+ (calcd for C22H22N2O3 383.1971).

NaBH4 reduction of alstonoxine C (9)

To a mixture of compound 9 (12.4 mg, 0.035 mmol) in MeOH (5 ml) at 0 °C was added NaBH4 (6.5 mg, 0.17 mmol). The solution was stirred at 0 °C for 1 h. Saturated Na2SO4 (5 ml) solution was added, and the product was extracted with CH2Cl2 (3 × 10 ml). The combined organic extract was dried (Na2SO4), filtered, and concentrated in vacuo, and the residue was purified by centrifugal preparative TLC (SiO2, 1% MeOH:CHCl3, NH3-saturated) to afford 11 (5.1 mg, 41%) and 11a (4.8 mg, 39%). Compound 11a: colorless oil; [α]D25 −33 (c 0.24, CHCl3); 1H NMR (CDCl3, 400 MHz) δ 7.69 (1H, d, J = 8.3 Hz), 6.58 (1H, dd, J = 8.3, 2.2 Hz), 6.45 (1H, d, J = 2.2 Hz), 4.02 (1H, m), 3.99 (1H, m), 3.96 (1H, m), 3.83 (3H, s), 3.24 (1H, m), 3.17 (3H, s), 2.76 (1H, m), 2.38 (1H, dd, J = 8.4, 2.3 Hz), 2.06 (1H, d, J = 8.4 Hz), 1.94 (1H, m), 1.77 (1H, m), 1.72 (1H, m), 1.58 (1H, m), 1.53 (1H, m), 1.29 (1H, d, J = 6 Hz); HRE- SIMS m/z 327.1718 (calcd for C19H28N2O3, 327.1703).

Determination of the C-19 configuration of compound 6 by Horeau’s method

To a solution of compound 9 (5 mg, 0.038 mmol) and anhydrous pyridine (1 ml), was added, racemic 2-phenylbutyric anhydride (0.1 ml). The resulting mixture was stirred for 24 h at rt. H2O (3 ml) was then added and the mixture was allowed to stand for 30 min. The pH of the solution was adjusted to pH 9 by dropwise addition of NaOH (0.1 M), after which the solution was extracted with CH2Cl2 (3 × 20 ml). The aqueous layer was acidified to pH 3 using 1.0 M HCl and extracted with CH2Cl2 (3 × 10 ml). Evaporation of the solvent from the organic phase gave the unreacted 2-phenylbutyric acid. The optical rotation of the unreacted 2-phenylbutyric acid was found to be negative (R), indicating the 5 configuration at C-19 in compound 11.

X-ray crystallographic analysis of alstonoxine C (9), alstonoxine B (12), and 2(S)-cathafoline (16)

X-ray diffraction analysis was carried out on a Bruker SMART APEX II CCD area detector system equipped with a graphite mono- chromator and a Mo Kα fine-focus sealed tube (λ = 0.71073 Å), at 100 K. The structure was solved by direct methods (SHELSX-97) and refined with full-matrix least-squares on F2 (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. Crystallographic data for compounds 9, 12, and 16 have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 0 1223 336033, or e-mail: deposit@ccdc.cam.ac.uk).

Crystallographic data of alstonoxine C (9): Colorless block crystals, C9H18N2O2, Mw = 376.44, orthorhombic, space group P212121, a = 7.3755(3) Å, b = 12.6137(3) Å, c = 19.5620(4) Å, V = 1819.93(8) Å3, Z = 100 K, R = 4%, Dcalc = 1.374 g cm−3, crystal size 0.16 × 0.18 × 0.24 mm³, F(000) = 704. The final R1 value is 0.0383 (wR2 = 0.0898) for 3750 reflections [I > 2σ(I)]. CCDC number: 935820.

Crystallographic data of alstonoxine B (12): Colorless block crystals, C9H18N2O2, Mw = 330.42, monoclinic, space group P21, a = 10.7388(4) Å, b = 10.5321(3) Å, c = 15.2354(5) Å, β = 92.851(2), V = 1721.02(10) Å³, Z = 100 K, R = 4%, Dcalc = 1.275 g cm−3, crystal size 0.21 × 0.41 × 0.49 mm³, F(000) = 712. The final R1 value is 0.0382 (wR2 = 0.1076) for 7729 reflections [I > 2σ(I)]. CCDC number: 935819.

Crystallographic data of 2(S)-cathafoline (16): Colorless block crystals, C21H26N2O2, Mw = 338.44, monoclinic, space group P21, a = 7.0991(3) Å, b = 8.6382(4) Å, c = 14.4902(6) Å, β = 90°, V = 971.55(7) Å³, Z = 100 K, R = 2%, Dcalc = 1.290 g cm−3, crystal size 0.08 × 0.26 × 0.37 mm³, F(000) = 364. The final R1 value is 0.0367 (wR2 = 0.0928) for 2138 reflections [I > 2σ(I)]. CCDC number: 936523.

Cytotoxicity assays

Cytotoxicity assays (Mosmann, 1983) were carried out following essentially the same procedure as described previously (Kam et al., 1998a, 2004b). Human oral epidermoid carcinoma cells (KB) and vincristine-resistant KB cells (VJ-300) were maintained in Eagle’s MEM, supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The cells were cultured at 37 °C under a humidified atmosphere in a CO2 incubator. The cells were then seeded in a 96-well microtiter plate (Nunc, Germany) at a concentration of 70,000 cells/ml and incubated in a CO2 incubator at 37 °C. After 24 h, the cells were treated with samples at six different concentrations (0.1, 0.3, 1, 3, 10 and 30 μg/ml) and incubated for 72 h. Wells containing untreated cells (without addition of sample) were regarded as negative controls. DMSO was used to dilute the samples and the final concentration of DMSO in each well was not in excess of 0.5% (v/v). No adverse effect due to presence of DMSO was observed. At the end of the incubation period, 20 μl of MTT working solution (5 mg MTT in 1 ml phosphate-buffered saline) was added into each well and the 96-well microtiter plate was incubated for another three hours at 37 °C. The medium was then gently aspirated from each well and 200 μl of DMSO was added to effect formazan solubilization. After agitation for 15 min, the absorbance of each well was measured with a micro plate reader (Emax, Molecular Devices, USA) at 540 nm with 650 nm. The cytotoxic activity of each sample was expressed as the IC50 value, which is the concentration of the test sample that causes 50% inhibition of cell growth. All the samples were assayed in three independent experiments.

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References


