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Alstilobanines A–E, new indole alkaloids from *Alstonia angustiloba*

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ABSTRACT

Five new alkaloids, alstilobanines A (**1**)–E (**5**) were isolated from *Alstonia angustiloba* (Apocynaceae) and their structures were determined by MS and 2D NMR spectral analysis. Alstilobanines A–E showed a moderate vasorelaxant activity against phenylephrine-induced contraction of isolated rat aorta.

1. Introduction

Alstonia species, which is widely distributed in the tropical regions of Africa and Asia, is a well-known rich source of unique heterocyclic alkaloids having a monoterpene indole skeleton and has attracted great interest from biogenetic[1] and biological points of view[2] such as anticancer, antibacterial, anti-inflammatory, antitussive, and antimalarial properties. Previous investigations have indicated that the existence of monoterpene indole alkaloids is related to plant inhabitability. For example, picrinine-type indole alkaloids have been isolated from generally found *Alstonia* species from India, Pakistan, and Thailand, whereas those belonging to the angustilobine skeleton exist predominantly in the plants from Indonesia and the Philippines.[3] In our search for structurally and biogenetically interesting indole alkaloids, five new alkaloids, alstilobanines A (**1**)–E (**5**) were isolated from *Alstonia angustiloba* (Apocynaceae) collected in Malaysia. In this paper, we describe the isolation, structure elucidation, and vasorelaxant activity of **1**–**5**.

1.1. Structures of alstilobanines A–E (1–5)

The leaves of *A. angustiloba* were extracted with MeOH, and the MeOH extract was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted to pH 9 with saturated Na_2CO_3, were extracted with $CHCl_3$. $CHCl_3$-soluble materials were subjected to a silica gel column ($CHCl_3$/MeOH 1:0 → 0:1). MeOH eluted fractions were purified by C_{18} HPLC (MeOH/H_2O/TFA solvent system) to afford alstilobanines A (**1**, 2.0 mg, 0.002%), B (**2**, 2.0 mg, 0.002%), C (**3**, 3.0 mg, 0.003%), D (**4**, 1.8 mg, 0.002%), and E (**5**, 2.5 mg, 0.003%) together with undulifoline (**6**),[4] 6,7-*seco*-angustilobine B (**7**),[3d] and alstonamic acid (**8**).[5]

Alstilobanine A (**1**) showed the pseudomolecular ion peak at *m/z* 345 $(M+H)^+$ in the ESIMS, and the molecular formula, $C_{19}H_{24}N_2O_4$, was established by HRFABMS [*m/z* 345.1809, $(M+H)^+$, Δ ± 0.0 mmu]. IR absorptions implied the presence of hydroxyl (3284 cm^{-1}) and ester carbonyl (1723 cm^{-1}) functionalities. ^1H and ^{13}C NMR data (Tables 1 and 2) revealed nineteen carbon signals due to four sp^2 quaternary carbons, four sp^2 methines, one ester carbonyl, two sp^3 quaternary carbons, two sp^3 methines, four sp^3 methylenes, one methyl, and one methoxy group. Among them, two methylenes (δ_C 53.6; δ_H 3.34 and δ_C 45.8; δ_H 3.02 and 3.38) and two quaternary carbons (δ_C 130.7 and 138.9) were ascribed to those bearing a nitrogen atom, while one methylene (δ_C64.4; δ_H 3.97 and 4.21), one methyl (δ_C53.3; δ_H 3.77), and two quaternary carbons (δ_C 71.8 and 175.5) were those bearing an oxygen atom.

Partial structures **a** (C-8 to C-11), **b** (C-18 and C-19), and **c** (C-3, C-13, and C-14) were deduced from detailed analysis of ^1H–^1H COSY spectrum of **1** (Fig. 1). The structure of the octahydroisoquinoline ring (C-3 to C-5, C-13 to C-15, C-19 to C-20, C-2, C-6, and N-4) was deduced from the HMBC correlations. The HMBC cross peaks of H-5, H-14, and H_3-18 to C-20, and H-5 and H-14 to C-19 indicated the connection among C-5, C-14, and C-19 through C-20. The connection between C-16 and C-21 through C-15 was deduced from HMBC correlations of H_2-16 to C-15 and C-21. And the connection to indole ring was elucidated by HMBC correlations for H_2-16 to C-2 and H_3-18 to C-6. Thus, the gross structure of alstilobanine A was elucidated to be **1** possessing a novel tetracyclic

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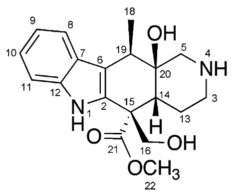
Table 1
^1H NMR Data [δ_H (*J*, Hz)] of Alstilobanines A (**1**)–E (**5**) in CD_3OD at 300 K

	1	**2**	**3**	**4**	**5**
3a	3.38 (br d, 12.5)	3.58 (dd, 13.8, 3.7)	3.01 (2H, m)	3.65 (2H, dd, 12.5, 3.5)	3.21 (m)
3b	3.02 (dd, 12.5, 12.2)	3.37 (dd, 13.8, 4.6)			3.19 (m)
5a	3.34 (2H, m)	4.90 (m)	4.75 (br s)	4.41 (d, 12.1)	2.96 (d, 13.1)
5b				4.05 (d, 12.6)	2.61 (d, 13.1)
6				6.29 (s)	
8	7.47 (d, 7.9)	7.69 (d, 7.9)	7.62 (d, 7.9)	7.48 (d, 7.9)	7.44 (d, 7.9)
9	7.02 (dd, 7.8, 7.2)	7.20 (dd, 7.7, 7.2)	7.09 (dd, 7.4, 7.4)	7.00 (dd, 7.3, 7.7)	7.04 (dd, 7.5, 7.4)
10	7.11 (dd, 7.1, 8.1)	7.25 (dd, 7.5, 7.5)	7.17 (dd, 7.7, 7.4)	7.09 (dd, 7.3, 7.8)	7.10 (dd, 7.8, 7.2)
11	7.32 (d, 8.2)	7.45 (d, 8.0)	7.40 (d, 8.1)	7.33 (d, 8.1)	7.47 (d, 8.1)
13a	2.07 (br d, 13.6)	2.54 (dddd, 15.0, 14.5, 4.6, 4.5)	2.15 (m)	1.97 (dddd, 14.1, 12.3, 12.0, 4.7)	1.72 (m)
13b	1.92 (m)	1.75 (br d, 15.8)	1.73 (br d, 15.0)	1.17 (dd, 14.3, 2.9)	1.19 (dddd, 14.1, 13.5, 13.3, 5.6)
14	2.74 (dd, 12.8, 4.0)	3.00 (br d, 3.0)	2.97 (d, 2.3)	3.57 (br d, 12.0)	2.74 (dd, 13.3, 5.2)
16a	4.21 (d, 11.6)	4.37 (d, 12.1)	4.35 (d, 12.1)	4.77 (m)	4.13 (d, 11.5)
16b	3.97 (d, 11.6)	4.04 (d, 12.1)	4.01 (d, 12.1)	4.22 (d, 12.0)	3.84 (d, 11.4)
18a	1.24 (d, 7.1)	3.70 (ddd, 13.0, 3.6, 3.6)	3.69 (ddd, 13.1, 3.5, 2.6)	4.40 (m)	4.31 (dd, 11.6, 3.1)
18b		3.64 (brdd, 12.0, 11.9)	3.60 (brdd, 12.2, 12.2)	4.34 (br d, 17.9)	3.57 (dd, 11.6, 3.1)
19a	3.19 (m)	2.12 (ddd, 12.1, 12.0, 2.2)	2.11 (m)	5.87 (br s)	3.18 (dd, 3.1, 3.1)
19b		1.19 (m)	1.26 (m)		
20		3.55 (m)	2.86 (m)		
OMe	3.77 (s)	3.82 (s)	3.79 (s)	3.71 (s)	3.90 (s)
NMe		3.40 (s)		3.48 (s)	

skeleton with a hydroxymethylene and a methoxycarbonyl at C-15, a methyl at C-19, and a hydroxyl at C-20. The relative stereochemistry of **1** was elucidated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 2). The presence of *cis* fused octahydroisoquinoline including stereochemistry at C-15 and C-19 was elucidated by NOESY correlations among H-14, H_2-16, and H_3-18 and between H-8 and H-19. The chair conformation of a piperidine ring was suggested by ^3J coupling constants ($^3J_{H\text{-}3/H\text{-}13}$ = 12.2 Hz), and NOESY correlations of H-3a to H-5a. Thus, the relative stereochemistry of **1** was assigned as shown in Figure 2.



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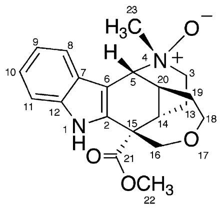

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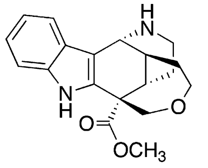

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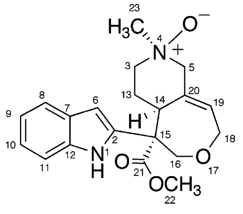

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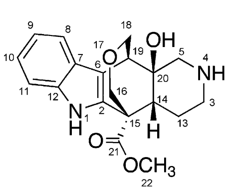

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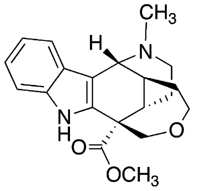

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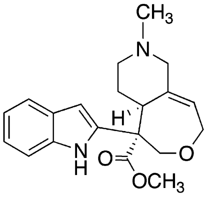

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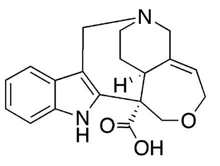

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Alstilobanine B (**2**) showed the pseudomolecular ion peak at *m*/*z* 357 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{24}N_2O_4$, was established by HRESIMS [*m*/*z* 357.1807, $(M+H)^+\Delta$ −0.2 mmu]. IR spectrum suggested the presence of carbonyl (1731 cm^{-1}) group. The ^{13}C NMR (Table 2) spectrum of **2** disclosed twenty carbon signals due to one ester carbonyl (δ_C 172.6), four sp^2 quaternary carbons (δ_C 140.5, 138.6, 128.0, and 104.0), one sp^3 quaternary carbon (δ_C 56.2), four sp^2 methines (δ_C 124.1, 122.0, 118.7, and 113.0), three sp^3 methines (δ_C 73.2, 37.3, and 34.7), five sp^3 methylenes (δ_C 79.0, 69.9, 58.7, 33.0, and 26.7), and two methyls (δ_C 56.4 and 53.2) attached to nitrogen and/or oxygen atoms. Proton and carbon signals for **2** were assigned by detailed analysis of the HSQC spectrum. The ^1H–^1H COSY spectrum revealed connectivities of C-8 to C-11 in unit **a** and C-3 to C-13, C-13 to C-14, C-14 to C-20, C-18 to C-20, and C-5 to C-20 in unit **b** (Fig. 3). HMBC correlations of H-8 (δ_H 7.69) to C-12 (δ_C 138.6) and C-6 (δ_C 104.0), H-11 (δ_H 7.45) to C-7 (δ_C 128.0), H-5 (δ_H 4.90) to C-2 (δ_C 140.5), C-6, and C-7, and H_2-16 (δ_H 4.37 and 4.04) to C-2, C-14 (δ_C 37.3), and C-15 (δ_C 56.2) revealed the presence of a tetrahydrocarbazole ring (C-2, C-14 to C-15, C20, C-5 to C-12, and N-1). The presence of an oxepane ring (C-14 to C-16, C-18 to C-20, and O-17) was deduced from the HMBC correlation of H_2-16 to C-18 (δ_C 69.9). HMBC correlations of H-16a and H_3-22 (δ_H 3.82) to C-21 (δ_C 172.6) implied a methoxy carbonyl group connected at C-15. Cross peaks of H_2-3 (δ_H 3.58 and 3.37) to C-5 (δ_C 73.2) and H_3-23 (δ_H 3.40) to C-3 and C-5 in the HMBC spectrum via the remaining oxygenated nitrogen atom implied the presence of an *N*-methylpiperidine *N*-

Table 2
^{13}C NMR Data (δ_C) of alstilobanines A (**1**)–E (**5**) in CD_3OD at 300 K

	1	**2**	**3**	**4**	**5**
2	130.7	140.5	138.8	135.2	135.3
3	45.8	58.7	37.0	67.5	39.8
5	53.6	73.2	51.7	75.9	49.0
6	115.9	104.0	104.2	101.2	112.5
7	127.7	128.0	127.1	129.1	126.4
8	118.7	118.7	118.8	121.1	118.0
9	119.9	122.0	120.8	120.7	120.7
10	123.1	124.1	123.5	123.0	122.4
11	112.3	113.0	112.4	112.2	113.0
12	138.9	138.6	138.6	138.0	137.8
13	22.3	26.7	28.9	25.5	23.4
14	37.4	37.3	38.5	45.5	51.5
15	53.7	56.2	55.9	57.3	56.2
16	64.4	79.0	78.9	71.9	75.4
18	18.8	69.9	69.8	70.1	67.2
19	39.0	33.0	33.5	135.2	43.9
20	71.8	34.7	38.3	129.1	75.6
COOMe	175.5	172.6	173.1	174.0	172.9
	53.3	53.2	53.0	53.1	53.2
NMe		56.4		57.3	

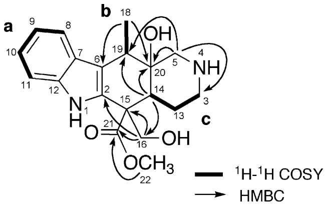


Figure 1. Selected 2D NMR correlations for alstilobanine A (**1**).

oxide ring (C-3, C-13 to C-14, C-20, C-5, N-4, and C-23). These data suggested that alstilobanine B possessed the rare uleine skeleton like undulifoline,[4] which has been isolated from *Alstonia undulifolia*. Thus, the structure of alstilobanine B was elucidated to be **2**.

The relative stereochemistry of **2** was elucidated by NOESY correlations as shown in Figure 4. NOESY correlations of H-5 to H-19b, H-20 to H-13a, H-14 to H-16b, H_3-22 to H-13b, and H_3-23 to H-5 indicated both a piperidine ring and a methoxy carbonyl group at C-15 were α-orientation, and both an oxepane ring and *N*-methyl were β-orientation. Thus, the relative stereochemistry of **2** was assigned as shown in Figure 4.

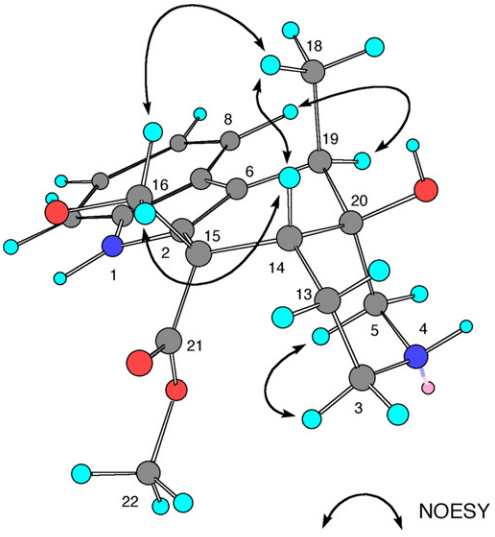


Figure 2. Selected NOESY correlations (arrows) and relative stereochemistry for alstilobanine A (**1**).

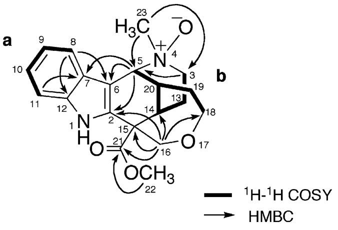


Figure 3. Selected 2D NMR correlations for alstilobanine B (**2**).

Alstilobanine C (**3**) showed the pseudomolecular ion peak at *m/z* 327 (M+H)$^+$ in ESIMS, and the molecular formula, $C_{19}H_{22}N_2O_3$, was established by HRESIMS [*m/z* 327.1702, (M+H)$^+$Δ −0.1 mmu], which was smaller than that of **2** by a 30 mu. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **3** were analogous to that of **2** except for the following observation: *N*-methyl signal (δ_H 3.40) lacking in **3** appeared for **2**. Detailed analysis of 2D NMR data (^{1}H–^{1}H COSY, HSQC, and HMBC) suggested that gross structure of **3** was the same as that of **2**except for *N*-oxide *N*-methyl group. The relative stereochemistry of **3** was deduced to be the same as those of **2** by NOESY correlations of H-5 to H-19β, H-14 to H-16β and H-18β, and H-20 to H-19α. Thus, alstilobanine C was assigned as **3**.

Alstilobanine D (**4**) showed the pseudomolecular ion peak at *m/z* 357 (M+H)$^+$ in ESIMS, and the molecular formula, $C_{20}H_{24}N_2O_4$, was established by HRESIMS [*m/z* 357.1806, (M+H)$^+$Δ −0.3 mmu]. The NMR data of **4** compared with those of 6,7-*seco*-angustilobine B (**7**),[3d] the similar chemical shift pattern except for that of around *N*-methyl group was observed. The presence of an *N*-oxide *N*-methyl group at *N*-4 was assigned by ^{13}C NMR chemical shifts of C-3 (δ_C 67.5), C-5 (δ_C 75.9), and C-23 (δ_C 57.3). The relative stereochemistry of *N*-methyl group of **4** was deduced to be α-orientation by a NOESY correlation between H-14 and H_3-23. Thus, alstilobanine D was assigned as **4**.

Alstilobanine E (**5**) showed the pseudomolecular ion peak at *m/z* 343 (M+H)$^+$ in ESIMS, and the molecular formula, $C_{19}H_{22}N_2O_4$, was established by HRESIMS [*m/z* 343.1651 (M+H)$^+$Δ −0.1 mmu]. ^{1}H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested the presence of one ester carbonyl, four sp^2 quaternary carbons, four sp^2 methines, five sp^3 methylenes, two sp^3 methines, two sp^3 quaternary carbons, and one methyl carbon. Among them, signals due to four nitrogen-bearing carbons at δ_C 39.8, 49.0, 135.3, and 137.8,

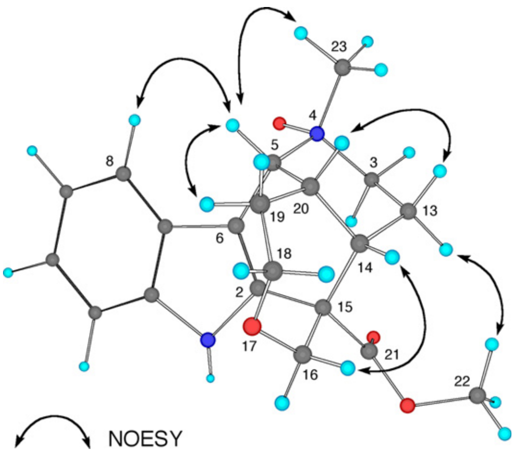


Figure 4. Selected NOESY correlations (arrows) and relative stereochemistry for alstilobanine B (**2**).

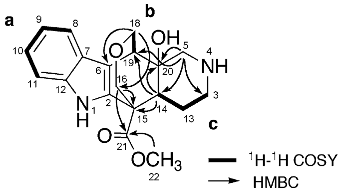


Figure 5. Selected 2D NMR correlations for alstilobanine E (**5**).

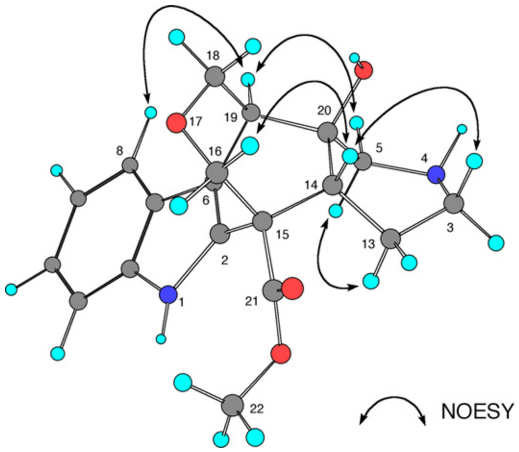


Figure 6. Selected NOESY correlations (arrows) and relative stereochemistry for alstilobanine E (**5**).

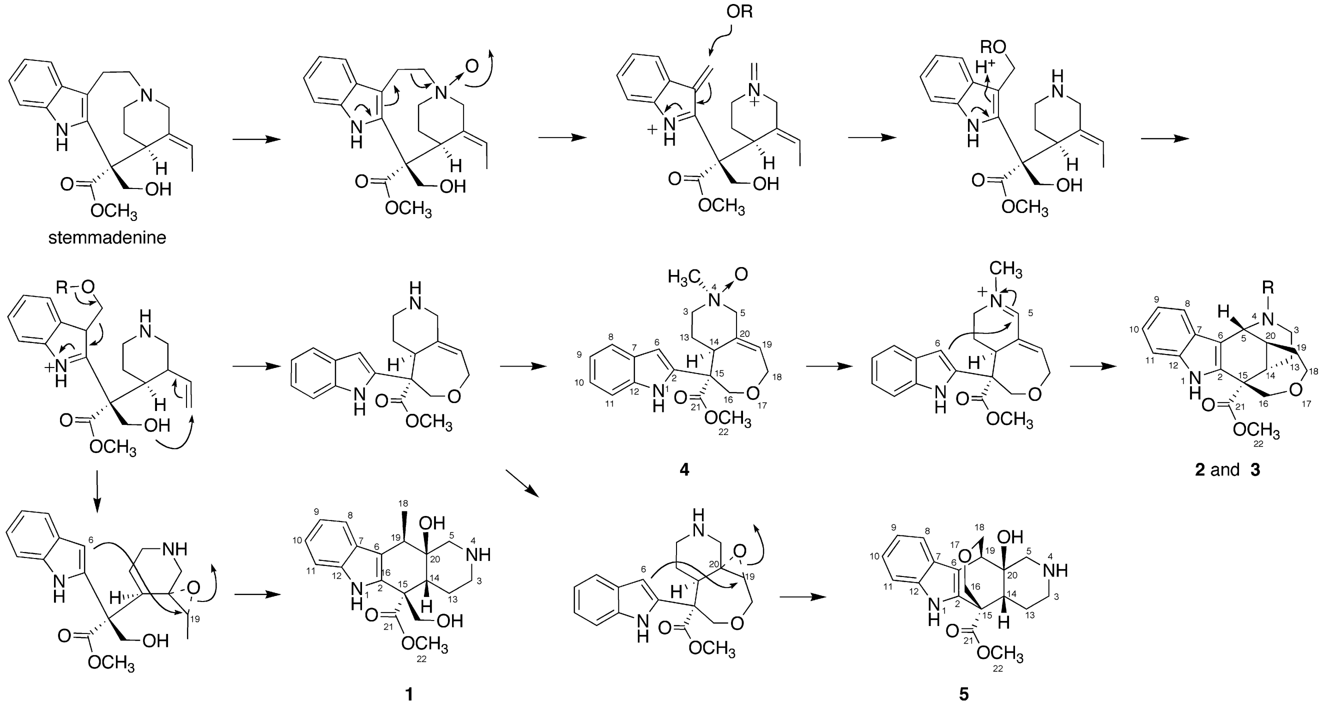
four oxygen-bearing carbons at δ_C 53.2, 67.2, 75.4, and 75.6 appeared. The molecular structure of **5** was deduced from extensive analyses of the two-dimensional NMR data, including the ^1H–^1H COSY, HSQC, and HMBC spectra. The ^1H–^1H COSY spectrum revealed connectivities of three partial structures **a** (C-8 to C-11), **b** (C-18 to C-19), and **c** (C-3 to C-13 and C-13 to C-14) as shown in Figure 5. Connectivities of C-5 and C-14 to C-19 through C-20-bearing a hydroxyl group were implied by HMBC correlations for H-5 and H-14 to C-20 (δ_C 75.6) and C-19 (δ_C 43.9). Cross peaks of H-5 to C-3 (δ_C 39.8) and C-14 (δ_C 51.5) in the HMBC spectrum *via* a nitrogen atom implied the presence of a piperidine ring. HMBC correlations were observed for H-18 to C-6 (δ_C 112.5) and C-16 (δ_C 75.4) suggesting that C-18 was connected to C-16 through an oxygen atom and C-19 was to C-6 of an indole ring. The presence of methoxy carbonyl group at C-15 was implied by HMBC cross peaks for H_3-22 and H_2-16 to C-21 (δ_C 172.9), and H-14 and H_2-16 to C-15 (δ_C 56.2). Thus, the molecular structure of alstilobanine E was elucidated to be **5** possessing a pentacyclic angustilodine skeleton without *N*-methyl group.[6]

The relative configuration of **5** was elucidated by NOESY correlations as depicted in the computer-generated three-dimensional drawing (Fig. 6). The presence of *cis* fused decahydroisoquinoline was elucidated by NOESY correlation of H-14 to H-16a and no correlation of H-5 to H-18. The boat conformation of a piperidine ring was suggested by ^3J coupling constants $^3J_{H_2-13/H-14} = 5.2$ and 13.3 Hz), and NOESY correlations of H-14 to H-3a, H-19 to H-5a, and H-5b to H-13b.

Biogenetic relationship among monoterpene indole alkaloids such as stemmadenine and 5-*nor*-indole derivatives such as vallesamine and apparicine has been discussed.[7] A plausible biogenetic path for a series of alstilobanines can be proposed in Scheme 1. Alstilobanines B (**2**) and C (**3**) possessing uleine skeleton and alstilobanine D (**4**) possessing *seco*-angustilobine B-type skeleton might be generated through *N*-oxidation of pericine-type indole alkaloid such as stemmadenine and pericine by Polonovski-type fragmentation[8] followed by recyclization, while alstilobanines A (**1**) and E (**5**) possessing angustilodine skeleton with and without an ether linkage might be derived from *seco*-angustilobine B-type skeleton through introduction of an epoxide at C-19 followed by cyclization and formation of C-6 to C-19 bond (Scheme 1).

1.2. Vasorelaxant activity

Alstilobanines A (**1**)–E (**5**), undulifoline (**6**), 6,7-*seco*-angustilobine B (**7**), and alstonamic acid (**8**) showed a slow relaxation activ-



Scheme 1. Plausible biogenetic formation of alstilobanines A (**1**)–E (**5**).

ity against phenylephrine (PE, 3×10^{-7} M)-induced contractions of thoracic rat aortic rings with endothelium (**1**: 44.3%; **2**: 21.2%; **3**: 28.0%; **4**: 10.0%; **5**: 6.8%; **6**: 33.3%; **7**: 7.0%; **8**: 35.0% at 3×10^{-5} M, respectively). Alstilobanine A (**1**) possessing angustilodine skeleton without an ether linkage showed more potent activity than alstilobanine E (**5**) with an ether linkage. Alstilobanines B (**2**), C (**3**), and undulifoline (**6**) with uleine skeleton showed a moderate activity, whereas alstilobanine D (**4**) and 6,7-*seco*-angustilobine B (**7**) with *seco* skeleton of alstonamic acid (**8**) showed a weak activity.[9]

2. Experimental

2.1. General methods

^{1}H and 2D NMR spectra were recorded on a Bruker Avance 600 spectrometer and chemical shifts were reported using residual CD_3OD (δ_H 3.31 and δ_C 49.0) as internal standards. HSQC experiments were optimized for $^1J_{CH}$ = 145 Hz and HMBC experiments for $^nJ_{CH}$ = 8 Hz. High resolution positive-mode ESI mass spectra were obtained on a Thermo Scientific LTQ Orbitrap XL spectrometer.

2.2. Materials

The leaves of *A. angustiloba* were collected at Malaysia in 2006. A voucher specimen has been deposited in the herbarium of Hoshi University.

2.3. Extraction and isolation

The leaves of *A. angustiloba* (100 g) were crushed and extracted with MeOH. The MeOH extract (7.5 g) was treated with 3% tartaric acid (pH 2) and then partitioned with EtOAc. The aqueous layer was treated with saturated Na_2CO_3 (aq) to pH 9 and extracted with $CHCl_3$ to give alkaloidal fraction (324 mg). The alkaloidal fraction was purified by SiO_2 column ($CHCl_3$/MeOH 1:0 → 0:1) and the fraction eluted by MeOH was purified by ODS HPLC (MeOH/H_2O/CF_3CO_2H, 10:90:0.1 → 55:45:0.1 → 100:0:0.1; flow rate, 3 mL/min; UV detection at 210 nm) to afford alstilobanines A (**1**, 0.002%), B (**2**, 0.002%), C (**3**, 0.003%), D (**4**, 0.002%), and E (**5**, 0.003%) together with known alkaloids, undulifoline (**6**, 0.006%),[4] 6, 7-*seco*-angustilobine B (**7**, 0.003%),[3d] and alstonamic acid (**8**, 0.003%).[5]

2.4. Alstilobanine A (1)

Colorless solid; $[\alpha]_D^{25}$ −13° (*c* 0.1, MeOH); IR (film) ν_{max} 3284, 2924, and 1723 cm^{-1}; UV (MeOH) λ_{max} 284 (ε 281) and 224 nm (ε 1235); ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 345 $(M+H)^+$; HRESIMS *m/z* 345.1809 $[(M+H)^+$, calcd for $C_{19}H_{25}N_2O_4$: 345.1809].

2.5. Alstilobanine B (2)

Colorless amorphous solid; $[\alpha]_D^{25}$ +18° (*c* 0.05, MeOH); IR (film) ν_{max} 3419, 2952, and 1731 cm^{-1}; UV (MeOH) λ_{max} 281 (ε 1908) and 226 nm (ε 2011); ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS m/*z* 357 $(M+H)^+$; HRESIMS *m/z* 357.1807 $[(M+H)^+$, calcd for $C_{20}H_{25}N_2O_4$: 357.1809].

2.6. Alstilobanine C (3)

Colorless amorphous solid; $[\alpha]_D^{25}$ −13° (*c* 0.07, MeOH); IR (film) ν_{max} 3420 cm^{-1}; UV (MeOH) λ_{max} 281 (ε 3006) and 228 nm (ε 2712); ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 327 $(M+H)^+$; HRESIMS *m/z* 327.1702 $[(M+H)^+$, calcd for $C_{19}H_{23}N_2O_3$: 327.1703].

2.7. Alstilobanine D (4)

Colorless amorphous solid; $[\alpha]_D^{25}$ +93° (*c* 0.05, MeOH); IR (film) ν_{max} 3734 and 2922 cm^{-1}; UV (MeOH) λ_{max} 282 (ε571) and 228 nm (ε 7008); ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 357. $(M+H)^+$; HRESIMS *m/z* 357.1806 $[(M+H)^+$, calcd for $C_{20}H_{25}N_2O_4$: 357.1809].

2.8. Alstilobanine E (5)

Colorless amorphous solid; $[\alpha]_D^{25}$ +16° (*c* 0.08, MeOH); IR (film) ν_{max} 3734 and 3445 cm^{-1}; UV (MeOH) λ_{max} 282 (ε 173) and 230 nm (ε 1860); ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 343 $(M+H)^+$; HRESIMS *m/z* 343.1651 $[(M+H)^+$, calcd for $C_{19}H_{23}N_2O_4$, 343.1652].

2.9. Vasodilation assay[9]

A male Wistar rat weighting 260 g was sacrificed by bleeding from carotid arteries under an anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, and modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations, 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, and 5% CO_2) bath of 5 mL KHS solution at 37 °C with one end connected to a tissue holder and the other to a force–displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M phenylephrine (PE). The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (ACh), and aortic ring in which 80% relaxation occurred was regarded as tissues with endothelium. When the PE-induced contraction reached a plateau, each sample was added.

These animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

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