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Alpneumines A–H, new anti-melanogenic indole alkaloids from *Alstonia pneumatophora*

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ABSTRACT

Eight new indole alkaloids, alpneumines A–H (**1**–**8**) were isolated from the Malaysian *Alstonia pneumatophora* (Apocynaceae) and their structures were determined by MS and 2D NMR spectroscopic methods. Alpneumines E and G (**5** and **7**), vincamine, and apovincamine showed anti-melanogenesis in B16 mouse melanoma cells.

1. Introduction

Alstonia species, which is widely distributed in the tropical regions of Africa and Asia, are a well-known rich source of unique heterocyclic alkaloids having a monoterpene indole skeleton and have 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 generally in *Alstonia* species from India, Pakistan, and Thailand, whereas those belonging to the angustilobine skeleton exist predominantly in the plants from Indonesia and Philippine.[3] In our search for structurally and biogenetically interesting indole alkaloids, eight new alkaloids, alpneumines A–H (**1**–**8**) were isolated from *Alstonia pneumatophora* (Apocynaceae) collected in Malaysia. In this paper, we describe the isolation and structure elucidation of **1**–**8**, and anti-melanogenesis with B16 mouse melanoma cells of **5** and **7**, vincamine, and apovincamine.

1.1. Isolation and structures of alpneumines A–H (1–8)

The leaves of *A. pneumatophora* 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 (EtOAc/MeOH 1:0→0:1). MeOH eluted fractions were purified by C_{18} HPLC (MeOH/H_2O/TFA solvent system) to afford alpneumines A (**1**, 1.0 mg, 0.0005%), B (**2**, 2.4 mg, 0.0012%), C (**3**, 1.0 mg, 0.0005%), D (**4**, 1.6 mg, 0.0008%), E (**5**, 1.0 mg, 0.0005%), F (**6**, 1.8 mg, 0.0009%), G (**7**, 3.4 mg, 0.0017%), and H (**8**, 0.8 mg, 0.0004%) together with known alkaloids, vincamine,[4,5] apovincamine,[5] alstilobanine B,[6] akuammidine,[7] scholarine,[8] echitamidine,[9] and actinophyllic acid.[10]

Alpneumine A {**1**, $[\alpha]_D^{20}$ + 101 (*c* 0.1, MeOH)} showed the pseudomolecular ion peak at *m/z* 371 $(M+H)^+$ in the LC–ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_5$, was established by LC–HRESIMS [*m/z* 371.1603 $(M+H)^+$, Δ +0.2 mmu]. ^{13}C NMR data (Table 2) revealed 20 carbon signals due to five sp^2 quaternary carbons, three sp^2 methines, one ketone, one ester carbonyl, one sp^3 quaternary carbon, three sp^3 methines, four sp^3 methylenes, one methyl, and one methoxy group. Among them, two methylenes (δ_C 66.6; δ_H 4.08, 4.11 and δ_C 60.1; δ_H 3.78, 3.85) and one methine (δ_C 75.5, δ_H 4.83) were ascribed to those bearing an N-oxide, while one methyl (δ_C 51.8; δ_H 3.77) and one quaternary carbon (δ_C 143.6) were those bearing an oxygen atom.

Partial structures **a** (C-9–C-11), **b** (C-5 and C-6), and **c** (C-3, C-14–C-15, and C-20–C-21) were deduced from detailed analysis of ^1H–^1H COSY spectrum of **1** (Fig. 1). The presence of a scholaricine skeleton was deduced from the HMBC correlations. Then, the HMBC cross-peaks of H_3-18 to C-19 and C-20 indicated the connection between C-20 and C-18 through C-19. In addition, chemical shifts of C-3, C-5, and C-21 implied the presence of an N-oxide at N-4.[11] Thus,

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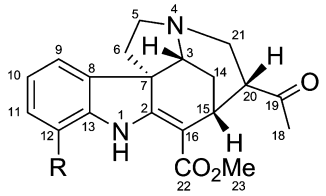

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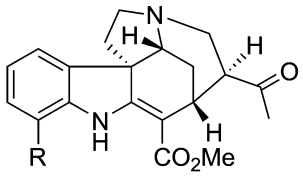

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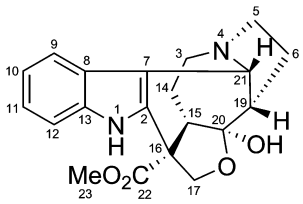
5: R=H



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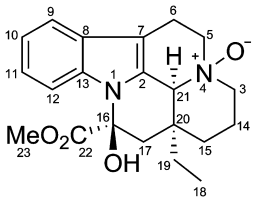

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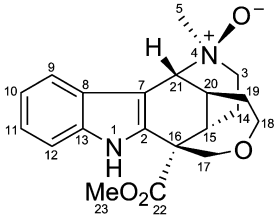

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Table 1
^{1}H NMR data [δ_H (*J*, Hz)] of alpneumines A–H (**1**–**8**) in CD_3OD at 303 K

	1	**2**	**3**	**4**	**5**
3	4.83 (m)	4.35 (br s)	4.53 (br s)	4.41 (br s)	4.33 (br s)
5a	4.11 (m)	3.45 (m)	3.84 (2H, m)	3.50 (m)	3.42 (m)
5b	4.08 (m)	3.20 (m)		3.30 (m)	3.24 (m)
6a	2.92 (m)	3.02 (m)	2.80 (m)	2.75 (m)	2.65 (m)
6b	2.37 (m)	2.06 (m)	2.19 (m)	2.14 (m)	2.08 (m)
9	7.06 (d, 7.6)	6.88 (d, 7.5)	7.41 (d, 7.2)	6.88 (dd, 7.4, 0.9)	7.31 (d, 7.5)
10	6.99 (dd, 8.0, 7.6)	6.83 (dd, 7.9, 7.5)	6.98 (dd, 7.7, 7.2)	6.84 (dd, 7.8, 7.4)	6.95 (dd, 7.9, 7.5)
11	6.89 (d, 8.0)	6.72 (d, 7.9)	7.21 (dd, 7.7, 7.5)	6.75 (dd, 7.8, 0.9)	7.20 (dd, 7.9, 7.7)
12			6.99 (d, 7.5)		7.00 (d, 7.7)
14a	2.58 (br d, 14.4)	2.21 (br d, 13.8)	2.58 (ddd, 14.2, 3.5, 2.5)	2.08 (ddd, 14.6, 3.3, 3.1)	2.11 (ddd, 14.5, 3.2, 3.2)
14b	1.71 (br d, 14.4)	1.60 (br d, 13.8)	1.59 (br d, 14.2)	1.38 (br d, 14.6)	1.32 (br d, 14.5)
15	3.83 (m)	3.64 (br s)	3.74 (m)	3.51 (m)	3.47 (m)
18	2.46 (3H, s)	2.35 (3H, s)	2.38 (3H, s)	2.36 (3H, s)	2.34 (3H, s)
20	3.73 (br d, 12.6)	3.16 (m)	3.54 (ddd, 12.5, 3.8, 3.1)	3.25 (m)	3.21 (m)
21a	3.85 (m)	3.13 (m)	3.80 (m)	3.63 (dd, 13.8, 6.0)	3.54 (m)
21b	3.78 (m)	3.02 (m)	3.41 (m)	3.11 (dd, 13.8, 5.7)	3.05 (dd, 13.9, 5.6)
23	3.77 (3H, s)	3.71 (3H, s)	3.72 (3H, s)	3.85 (3H, s)	3.83 (3H, s)

	6	**7**	**8**
3a	3.28 (m)	3.54 (m)	3.40 (dd, 13.5, 2.7)
3b	3.04 (br d, 13.9)	3.28 (m)	2.87 (m)
5[a]	3.72 (m)	4.17 (m)	3.10 (3H, s)
5b	3.62 (m)	3.96 (m)	
6a	2.75 (m)	3.23 (2H, m)	
6b	2.51 (m)		
9	7.62 (d, 7.9)	7.52 (d, 7.7)	7.64 (d, 7.8)
10	7.15 (dd, 7.9, 7.4)	7.15 (dd, 7.7, 7.0)	7.15 (dd, 7.8, 7.1)
11	7.21 (dd, 7.9, 7.4)	7.19 (dd, 8.1, 7.0)	7.20 (dd, 7.9, 7.1)
12	7.43 (d, 7.9)	7.24 (d, 8.1)	7.40 (d, 7.9)
14a	2.43 (m)	2.43 (m)	2.60 (dddd, 14.9, 14.4, 4.1, 4.0)
14b	2.29 (m)	1.61 (m)	1.56 (br d, 14.9)
15a	2.92 (br d, 5.7)	2.02 (m)	2.89 (br s)
15b		1.64 (m)	
17a	4.40 (d, 8.0)	2.40 (d, 15.0)	4.33 (d, 12.1)
17b	3.72 (d, 8.0)	2.25 (d, 15.0)	4.01 (d, 12.1)
18a		1.03 (3H, t, 7.5)	3.68 (ddd, 12.6, 3.5, 3.4)
18b			3.62 (dd, 12.6, 12.4)
19a	3.22 (m)	2.41 (m)	2.07 (m)
19b		2.06 (m)	1.15 (m)
20			3.73 (m)
21a	5.48 (d, 7.4)	4.80 (m)	4.27 (br s)
21b			
23	3.92 (3H, s)	3.78 (3H, s)	3.79 (3H, s)

Table 2
^{13}C NMR data (δ_C) of alpneumines A–H (**1**–**8**) in CD_3OD at 303 K

	1	2	3	4	5
2	168.3	171.2	168.6	168.4	168.4
3	75.5	62.3	75.7	62.0	61.5
5	66.6	53.9	68.0	53.7	53.8
6	40.2	42.5	40.4	42.7	44.0
7	53.8	56.7	54.0	57.4	57.8
8	134.5	135.9	133.8	135.1	134.3
9	112.3	112.3	120.9	112.6	121.3
10	124.2	123.8	122.8	123.9	122.5
11	117.6	117.0	130.2	117.3	129.8
12	143.6	143.3	111.9	143.4	111.6
13	132.5	132.7	145.6	132.8	145.9
14	27.9	31.0	28.1	25.5	26.0
15	30.3	30.9	30.9	28.2	28.5
16	98.1	97.6	97.8	102.1	102.1
18	29.6	29.6	29.7	28.4	28.7
19	207.7	209.3	208.6	210.5	210.9
20	46.0	49.0	46.5	48.6	48.9
21	60.1	46.7	60.9	46.7	47.0
22	168.0	168.5	168.0	168.4	168.4
23	51.8	51.6	51.7	52.0	51.8

	6	7	8
2	138.4	127.2	139.3
3	47.0	58.6	59.5
5	50.7	69.7	58.9
6	26.1	20.5	
7	103.4	106.4	106.8
8	128.3	128.4	128.4
9	118.2	119.7	118.9
10	121.4	122.0	121.4
11	123.9	124.2	123.5
12	112.6	112.9	112.7
13	137.5	137.0	138.5
14	19.7	17.4	27.0
15	55.9	25.9	38.1
16	58.6	83.2	56.2
17	74.8	44.4	79.0
18		8.3	70.2
19	53.4	31.6	33.4
20	107.8	38.4	34.6
21	62.4	72.8	73.7
22	171.5	173.8	173.1
23	53.5	54.1	53.0

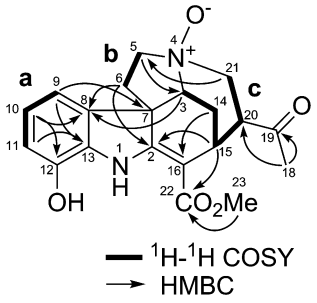

Figure 1. Selected 2D NMR correlations for alpneumine A (**1**).

the gross structure of alpneumine A was elucidated to be **1** possessing a scholaricine skeleton with an acetyl group at C-20 and an N-oxide at N-4. The relative stereochemistry of **1** was elucidated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 2). The presence of an acetyl group with α-configuration at C-20 was elucidated by NOESY correlations between H-20 and H-14a. The NOESY correlation of H-15/H-20 and coupling pattern of H-14a (br d, 14.4 Hz) indicated that both H-3 and H-15 were equatorially oriented in piperidine ring (C-3, C-14–C-15, C-20–C-21, and N-4). The conformation of *cis*-octahydroindolizine ring was supported by the NOESY correlation of H-6a/H-21a. Thus, the relative stereochemistry of **1** was assigned as shown in Figure 2.

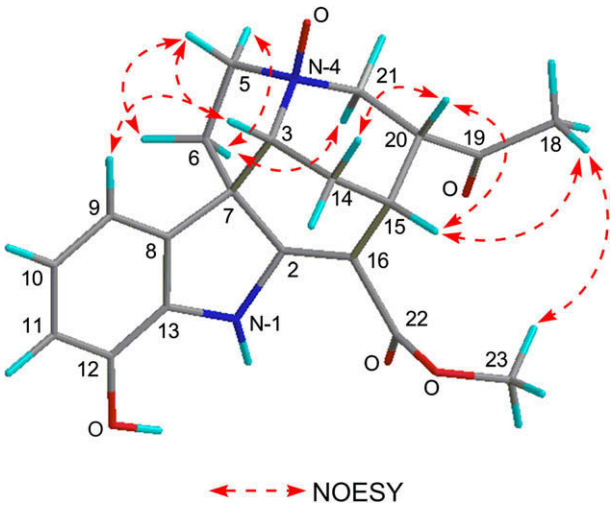

Figure 2. Selected NOESY correlations (arrows) and relative stereochemistry for alpneumine A (**1**).

Alpneumine B (**2**) showed the pseudomolecular ion peak at *m/z* 355 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_4$, was established by HRESIMS [*m/z* 355.1651 $(M+H)^+$, Δ −0.1 mmu], which was smaller than that of **1** by a 16 Da. IR spectrum suggested the presence of hydroxyl (3434 cm^{-1}) group. The ^1H and ^{13}C NMR data (Tables 1 and 2) of **2** disclosed 20 carbon signals that were similar to those of **1** except for the chemical shifts of C-3, C-5, and C-21. These results showed that **2** did not possess an N-oxide moiety. A detailed analysis of 2D NMR data (^1H–^1H COSY, HSQC, and HMBC) suggested that the gross structure of **2** was the same as that of **1** except for an N-oxide group. The relative stereochemistry of **2** was elucidated by the NOESY correlations. NOESY correlation of H-14a to H-20 indicated that an acetyl group at C-20 was α-configuration as that of **1**. Treatment of alpneumine A (**1**) with Na_2SO_3 in aqueous MeOH afforded the reductive derivative, whose spectroscopic data and specific rotation were identical with alpneumine B (**2**). Thus, the configuration of **2** was assigned as the same as **1**.

Alpneumine C (**3**) showed the pseudomolecular ion peak at *m/z* 355 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_4$, was established by HRESIMS [*m/z* 355.1653 $(M+H)^+$, Δ +0.1 mmu], which was smaller than that of **1** by a 16 Da. The ^1H and ^{13}C NMR data (Tables 1 and 2) of **3** were analogous to those of **1** except for ^1H and ^{13}C chemical shifts around the C-12 sp^2 methine. A detailed analysis of 2D NMR data suggested that the gross structure of **3** was the same as that of **1** except for the presence of a hydroxyl group at C-12. The conformation of a piperidine ring (C-3, C-14–C-15, C-20–C-21, and N-4) was deduced to be a chair form by a NOESY correlation of H-14a to H-20 and ^3J coupling constant ($^3J_{20,21a}$ = 12.5 Hz). Thus, alpneumine C was assigned as **3**.

Alpneumine D (**4**) showed the pseudomolecular ion peak at *m/z* 355 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_4$, was established by HRESIMS [*m/z* 355.1653 $(M+H)^+$, Δ +0.1 mmu]. The NMR data of **4**, when compared with those of **2**, the similar chemical shift pattern except for those of C-14 and C-15 was observed. Furthermore, ^3J coupling constants ($^3J_{20,21a}$ = 6.0 Hz, $^3J_{20,21b}$ = 5.7 Hz) was difference from those of **2**. This phenomenon indicated that the relative stereochemistry at C-20 was different from **2**. As a result of comparison of the chemical shifts of C-14 and C-15 of **4** with those of **2** and 19-epischolaricine,[3c] **4** showed similar ^{13}C chemical shifts to those of 19-epischolaricine (δ_{C14} 28.2, δ_{C15} 27.9). Furthermore, a NOESY correlation between H-14a and H-20 was not observed. From these results, alpneumine D was an epimer at C-20 of alpneumine B.

Alpneumine E (**5**) showed the pseudomolecular ion peak at *m/z* 339 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_3$, was

established by HRESIMS [*m/z* 339.1703 $(M+H)^+$, Δ 0.0 mmu]. ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested that the structure of **5** was the analogue to **4** except for that around C-12. Chemical shift at C-12 of **5** shifted upfield as compared with **4** and this indicated that C-12 of **5** did not possess hydroxyl moiety. The relative stereochemistry at C-20 of **5** was identical with that of **4,** judging by comparing of chemical shifts around C-20 and no NOESY correlation between H-14a and H-20.

Alpneumine F (**6**) showed the pseudomolecular ion peak at *m/z* 355 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}N_2O_4$, was established by HRESIMS [*m/z* 355.1648 $(M+H)^+$, Δ −0.4 mmu], which was larger than that of actinophyllic acid[10] by a CH_2 unit. ^1H and ^{13}C NMR data of **6** were analogous to those of actinophyllic acid, although a methoxy signal (δ_H 3.92) correlating with C-22 (δ_C 171.5) in HMBC spectrum was observed. Therefore, the structure of **6** was assigned to be C-22 methyl ester of actinophyllic acid.

Alpneumine G (**7**) showed the pseudomolecular ion peak at *m/z* 371 $(M+H)^+$ in ESIMS, and the molecular formula, $C_{21}H_{26}N_2O_4$, was established by HRESIMS [*m/z* 371.1967 $(M+H)^+$, Δ +0.2 mmu]. ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested that the structure of **7** was the analogue to vincamine.[4] Comparing chemical shifts of **7** and vincamine suggested to be an N-oxide of vincamine. Treatment of alpneumine G (**7**) with Na_2SO_3 in aqueous MeOH afforded the reductive derivative, whose spectroscopic data and specific rotation were identical with vincamine.

Alpneumine H (**8**) 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* 357.1803 $(M+H)^+$, Δ −0.6 mmu]. The NMR data suggested that the structure of **8** coincided with alstilobanine B with a rare uleine skeleton like undulifoline,[12] which has been isolated from *Alstonia undulifolia*, except for chemical shifts around N-4.[6] The chemical shifts of H_2-3 and H-21 of **8** were observed at higher field in comparison with those of alstilobanine B, which was indicated that a shielding effect by N-4-oxide in alstilobanine B was stronger than those of **8** (Fig. 3). Furthermore, the presence of NOESY correlations of H_3-5/H-9 and H-21 indicated that **8** and alstilobanine B were stereoisomers at an N-4-oxide (Fig. 4).

Biogenetic relationship among monoterpene indole alkaloids such as stemmadenine and 5-*nor*-indole derivatives such as vallesamine and apparicine have been discussed.[13] Alpneumines F (**6**) and H (**8**) possessing an uleine skeleton might be generated through N-oxidation of a pericine-type indole alkaloid such as stemmadenine and pericine by Polonovski-type fragmentation,[14] while alpneumines A–E (**1**–**5**) possessing a scholaricine skeleton[8] might be derived from pre-acuamicine. Alpneumine G (**7**) might be produced by rearrangement from an aspidosperma skeleton.[15]

1.2. Anti-melanogenesis activity

Skin pigmentation is the result of melanogenesis that occurs in melanocytes and/or melanoma cells.[16] The regulation of cellular pigmentation can be controlled at many different stages of melanogenesis. Especially, tyrosinase inhibitors and antioxidants can be used for inhibition of cellular pigmentation[17] since tyrosinase is a key enzyme[18] to produce melanin, and a copper-containing enzyme that can catalyze two different reactions: the hydroxylation of L-tyrosine to DOPA; and the conversion of DOPA to DOPA quinones.[19] These quinones lead to polymeric pigmented materials by enzymatic and non-enzymatic reactions, resulting in the pigment deposition on skin.[20] Inhibition of melanin synthesis is important for the treatment of skin pigmentations, whitening reagent as a cosmetics.[21]

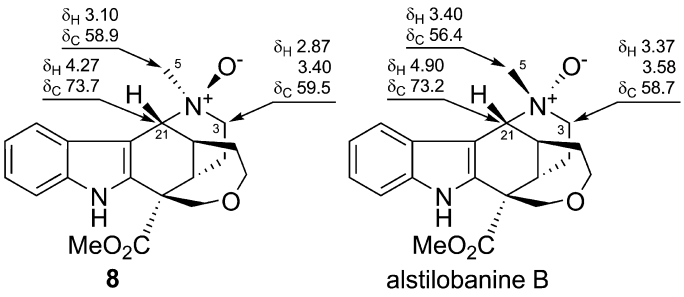

Figure 3. Chemical shifts of alpneumine H (**8**) and alstilobanine B. Left: alpneumine H, Right: alstilobanine B.

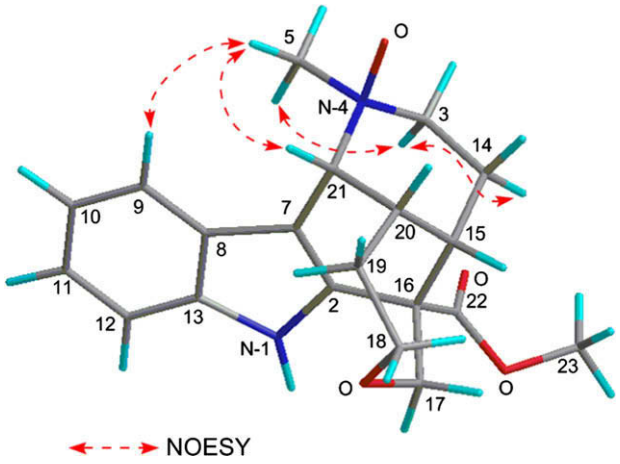

Figure 4. Selected NOESY correlations (arrows) and relative stereochemistry for alpneumine H (**8**).

B16 melanoma cells stimulated by IBMX, melanin inducer (3-isobutyl-1-methylxanthine), were cultured in the presence of isolated alkaloids at 6.3–100 μM for 4 days. Figure 4 showed the melanin content and the cell viability treated with each compound. After the treatment for 4 days, melanin content in B16 melanoma cells was determined by measuring OD at 400 nm, and the cell viability also evaluated by MTT method. Alpneumine G (**7**), vincamine, and apovincamine belonging to an eburnane type skeleton markedly decreased the melanin content (<50%) in B16 cells at high cell viability dose dependently (IC_{50} 58.3 μM for **7**, 68.9 μM for vincamine, and 49.8 μM for apovincamine). There was no tendency between oxidative stages of the eburnane type skeleton about melanogenesis inhibitory activity on B16 melanoma cell. Although alpneumine G (**7**) showed the efficient activity on anti-melanogenesis, it showed low cytotoxicity at high dose. Among alpneumines A–E (**1**–**5**) belonging to the scholaricine type skeleton, alpneumine E (**5**) only showed moderate inhibition of melanogenesis (IC_{50} 71.4 μM). It was the first report to find this activity for eburnane type indole alkaloids such as alpneumine G (**7**) and vincamine.

Inhibition of melanogenesis can be achieved by antioxidation and inhibition of tyrosinase. The effect of these alkaloids on melanogenesis by tyrosinase was examined using in vitro mushroom tyrosinase assay.[22,23] However, none of them showed the tyrosinase inhibition at even high dose (IC_{50} >100 μM). To determine the effect on cellular tyrosinase protein level in B16 melanoma cells, we performed Western blotting analysis on cell lysate obtained from B16 melanoma cells after treatment of hit compounds. Alpneumine G (**7**) was incubated with 0.1 mM IBMX in B16 melanoma cells. As shown in Figure 5, alpneumine G decreased the tyrosinase expression levels in B16 cells at 50 and 75 μM, respectively. Therefore, alpneumine G (**7**) decreased the tyrosinase protein expression, resulting in the reduction of melanin content in B16 melanoma cells. The mode of action of **7** might be due to inhibition of tyrosinase expression in B16 melanoma cells (Fig. 6).

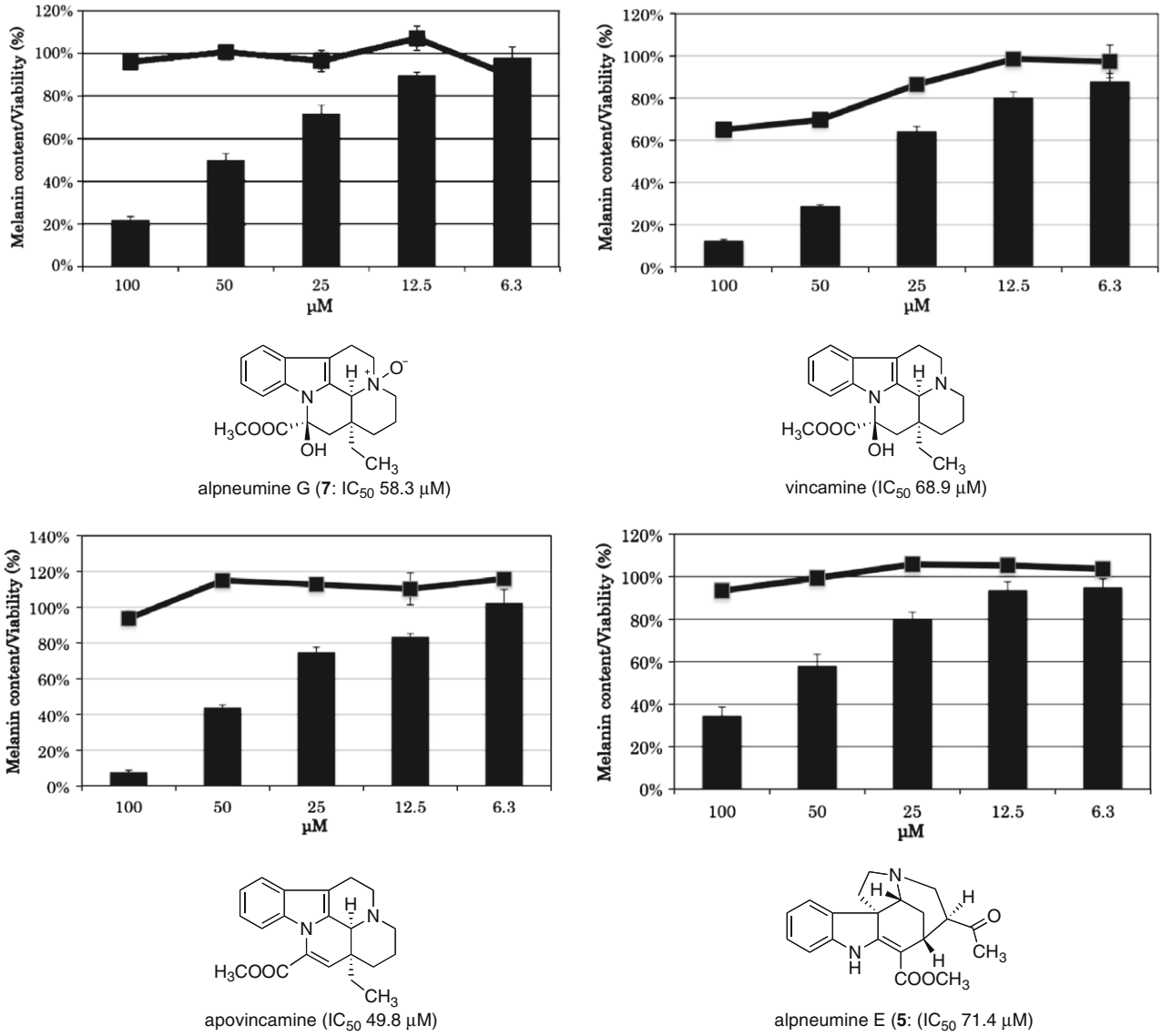

Figure 5. Melanin content in B16 melanoma cells and cell viability (–■–) treated with alpneumine E and G (**5** and **7**), vincammine, and apovincamine with IBMX.

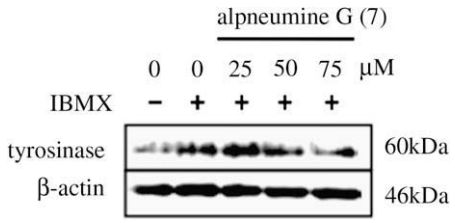

Figure 6. Effect of alpneumine G (**7**) on tyrosinase protein expression in B16 melanoma cells.

2. Experimental section

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. Material

The leaves of *A. pneumatophora* were collected at Malaysia in 2006. A voucher specimen (FR050802) has been deposited in the herbarium of Hoshi University.

2.3. Extraction and isolation

The leaves of *A. pneumatophora* (200 g) were crushed and extracted with MeOH. The MeOH extract (57.8 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 (1.2 g). The alkaloidal fraction was purified by SiO_2 column (AcOEt/MeOH 1:0→0:1) and the fraction eluted by MeOH was purified by ODS HPLC (MeOH/H_2O/TFA, 10:90:0.1→55:45:0.1→100:0:0.1; flow rate, 3 mL/min;

UV detection at 254 nm) to afford alpneumines A (**1**, 0.0005%), B (**2**, 0.0012%), C (**3**, 0.0005%), D (**4**, 0.0008%), E (**5**, 0.0005%), F (**6**, 0.0009%), G (**7**, 0.0017%), and H (**8**, 0.0004%) together with known alkaloids, vincamine (0.0055%),[4] apovincamine (0.0030%),[5] alstilobanine B (0.0005%),[6] akuammidine (0.0020%),[7] scholarine (0.0009%),[8] echitamidine (0.0010%),[9] and actinophyllic acid (0.0002%).[10]

2.3.1. Alpneumine A (1)

Colorless amorphous solid; $[\alpha]_D^{20}+101$ (*c* 0.1, MeOH); IR (film) ν_{max} 3434 and 1638 cm^{-1}; UV (MeOH) λ_{max} 335 (ε 2930) and 233 nm (ε 4170); ^1H and ^{13}C NMR data (Table 1 and Table 2); ESIMS *m*/*z* 371 $(M+H)^+$; HRESIMS *m*/*z* 371.1603 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_5$: 371.1601].

2.3.2. Alpneumine B (2)

Colorless amorphous solid; $[\alpha]_D^{20}+20$ (*c* 0.1, MeOH); IR (film) ν_{max} 3434 and 1638 cm^{-1}; UV (MeOH) λ_{max} 335 (ε 3010) and 232 nm (ε 3490); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 355 $(M+H)^+$; HRESIMS *m*/*z* 355.1651 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_4$: 355.1652].

2.3.3. Alpneumine C (3)

Colorless amorphous solid; $[\alpha]_D^{20}+25$ (*c* 0.1, MeOH); IR (film) ν_{max} 3418 and 1645 cm^{-1}; UV (MeOH) λ_{max} 327 (ε 2300), 292 (ε 1970), and 226 nm (ε 2860); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 355 $(M+H)^+$; HRESIMS *m*/*z* 355.1653 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_4$: 355.1652].

2.3.4. Alpneumine D (4)

Colorless amorphous solid; $[\alpha]_D^{20}+21$ (*c* 0.1, MeOH); IR (film) ν_{max} 3434 and 1644 cm^{-1}; UV (MeOH) λ_{max} 334 (ε 3460) and 233 nm (ε 4160); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 355 $(M+H)^+$; HRESIMS *m*/*z* 355.1653 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_4$: 355.1652].

2.3.5. Alpneumine E (5)

Colorless amorphous solid; $[\alpha]_D^{20}+13$ (*c* 0.1, MeOH); IR (film) ν_{max} 3360, 2951, 2926, 1681, and 1603 cm^{-1}; UV (MeOH) λ_{max} 326 (ε 2870), 291 (ε 2310), and 225 nm (ε 4330); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 339 $(M+H)^+$; HRESIMS *m*/*z* 339.1703 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_3$: 339.1703].

2.3.6. Alpneumine F (6)

Colorless amorphous solid; $[\alpha]_D^{20}+73$ (*c* 0.1, MeOH); IR (film) ν_{max} 3417 and 1675 cm^{-1}; UV (MeOH) λ_{max} 281 (ε 1670) and 217 nm (ε 7130); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 355 $(M+H)^+$; HRESIMS *m*/*z* 355.1648 $[(M+H)^+$, calcd for $C_{20}H_{23}N_2O_4$: 355.1652].

2.3.7. Alpneumine G (7)

Colorless amorphous solid; $[\alpha]_D^{20}+109$ (*c* 0.1, MeOH); IR (film) ν_{max} 3649, 2953, 1748, and 1684 cm^{-1}; UV (MeOH) λ_{max} 267 (ε 1070) and 221 nm (ε 4540); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 371 $(M+H)^+$; HRESIMS *m*/*z* 371.1967 $[(M+H)^+$, calcd for $C_{21}H_{27}N_2O_4$: 371.1965].

2.3.8. Alpneumine H (8)

Colorless amorphous solid; $[\alpha]_D^{20}+106$ (*c* 0.1, MeOH); IR (film) ν_{max} 3735, 3648, and 1682 cm^{-1}; UV (MeOH) λ_{max} 281 (ε 1220) and 219 nm (ε 5150); ^1H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 357 $(M+H)^+$; HRESIMS *m*/*z* 357.1803 $[(M+H)^+$, calcd for $C_{20}H_{25}N_2O_4$: 357.1809].

2.4. Chemical conversion of alpneumine A (1) into alpneumine B (2)

To a solution of alpneumine A (**1**, 0.2 mg) in aqueous MeOH (0.2 mL) was added Na_2SO_3 (1.0 mg) and the mixture was kept at rt for 30 min. After evaporation, the residue was applied to a silica gel column ($CHCl_3$/MeOH, 4:1) to give a compound (0.15 mg), whose spectroscopic data and $[\alpha]_D$ value were identical with those of natural alpneumine B (**2**).

2.5. Chemical conversion of alpneumine G (7) into vincamine

To a solution of alpneumine G (**7**, 0.2 mg) in aqueous MeOH (0.2 mL) was added Na_2SO_3 (1.0 mg) and the mixture was kept at rt for 30 min. After evaporation, the residue was applied to a silica gel column ($CHCl_3$/MeOH, 4:1) to give a compound (0.15 mg), whose spectroscopic data and $[\alpha]_D$ value were identical with those of natural vincamine.

2.6. Anti-melanogenesis assay

B16 cells were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin. B16 cells at 5000 cells in 100 μL per well were seeded onto 96-well microtiter plate, and were preincubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the medium containing IBMX (100 μM) with or without the test sample of different concentrations for 48 h, and then the medium were replaced the same condition fresh medium, further incubation for 48 h. The cells were dissolved in 50 μL of 1 N NaOH, and incubated at 90 °C for 1 h. Relative melanin content was determined spectrophotometrically by absorbance at 400 nm. Arbutin was used as a positive control.

2.7. Cell viability assay

The cell viability was determined by MTT assay. 15 μL of MTT solution (5 mg/mL) was added into each well of the cultured medium. After further 2 h incubation, the medium was removed, and then 50 μL of DMSO were added to resolve the formazan crystals. The optical density measurements were made using a microplate reader equipped with a two wavelengths system at 550 nm and 700 nm. In each experiment, three replicates were prepared for each sample. The ratio of the living cells was determined on the basis of the difference of the absorbance between those of samples and controls.

2.8. Western blotting

Cells treated with sample were lysated in lysis buffer, and extracts were separated on a 7.5% SDS–PAGE. Proteins were transferred to an Immuno-Blot polyvinylidene difluoride membrane (PVDF, Bio-Rad) and blocked for 1 h with 5% skim milk. Blotted membranes were incubated with primary antibodies diluted 1:2000 for anti-tyrosinase (Santa Cruz) for overnight, 1:2000 for β-actin (Sigma) for 1 h, followed by horseradish peroxide–conjugated rabbit or mouse IgG (Amersham). Protein bands were visualized with ECL solution (GE Healthcare).

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