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Pyridoacridine Alkaloids Inducing Neuronal Differentiation in a Neuroblastoma Cell Line, from Marine Sponge *Biemna fortis*

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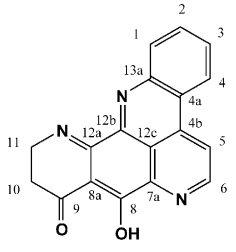
Abstract—A new and three known pyridoacridine alkaloids were isolated from the Indonesian marine sponge *Biemna fortis* as neuronal differentiation inducers against a murine neuroblastoma cell line, Neuro 2A. The chemical structure of the new compound, labuanine A (**1**), was determined by spectroscopic study and chemical conversion. These pyridoacridine alkaloids induced multipolar neuritogenesis in more than 50% of cells at 0.03–3 μM concentration. Compound **3**, which showed the strongest neuritogenic activity among them, also induced increase of acetylcholinesterase, a neuronal marker in Neuro 2A and arrested cell cycle at the G2/M phase.

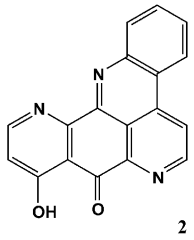
Introduction

Substances inducing differentiation against tumor cells are expected to be new candidates for cancer chemotherapy, since tumor cells retain differentiation capability in many cases, and these substances may exhibit selective activity against tumor cells and weak cytotoxicity against normal cells. In fact, clinical trials are in progress to evaluate the efficacy of retinoids, which induce differentiation against various leukemia or neuroblastoma cell lines, in the differentiation therapy of cancers.[1,2] In addition, new differentiation inducers (e.g., lactacystin,[3] epolactaene,[4] and staurosporin[5]) are used as bioprobes for studying the mechanism of differentiation. In spite of these extensive studies, details of the differentiation process in tumor cells are still unclear.

Marine organisms are rich sources of bioactive secondary metabolites, some of which are candidates for anticancer drugs.[6] In the course of our study of bioactive substances from marine organisms, we found a neuritogenic long-chain polyketide having a terminal 1-yn-3-ol moiety, lembehyne A, from an Indonesian marine sponge of *Haliclona* sp.[7] We further isolated a new and three known pyridoacridine alkaloids from the Indonesian marine sponge *Biemna fortis* as neuronal differentiation inducers against a murine neuroblastoma cell line, Neuro 2A. This paper presents the isolation, structure elucidation, and biological activity of these pyridoacridine alkaloids (Chart 1).



labuanine A (**1**)



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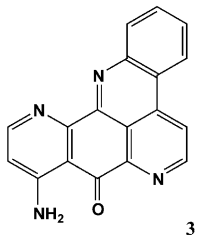

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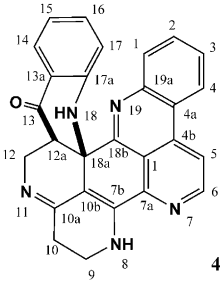

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Chart 1.

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Results and Discussion

The MeOH extract of the Indonesian marine sponge *B. fortis* showed neuritogenic activity against Neuro 2A cells at 10 μg/mL concentration. In the preliminary examination, the alkaloid fraction was found to exhibit neuritogenic activity. The MeOH extract was partitioned into an EtOAc–5% aq HCl mixture, and the 5% aq HCl-soluble portion was further partitioned into an EtOAc–water mixture after neutralization with aq NaOH. The resulting EtOAc soluble portion was subjected to reversed phase HPLC separation to furnish a new pyridoacridine alkaloid, labuanine A (**1**) (0.007% from the MeOH extract) and three known related alkaloids, **2**, **3**, and **4**. The positive FAB MS of **1** gave a quasi-molecular ion [$(M+H)^+$] peak at *m/z* 302 and the molecular formula of **1** was determined as $C_{18}H_{12}N_3O_2$ by HR-positive FABMS and NMR analysis. The ^{1}H NMR spectrum of **1** showed signals ascribable to six aromatic protons (δ 9.30, 9.18, 9.09, 8.41, 8.18, and 8.11) and two methylenes (δ 4.11, 2H, t, $J=7.6$ Hz and δ 2.93, 2H, t, $J=7.6$ Hz). The UV absorption maxima (λ_{max} at 357, 317, 288 nm) of **1** suggested that **1** has a long conjugated chromophore. The presence of a conjugated ketone group was readily revealed by the characteristic ^{13}C NMR signals at δc 193.5 and the absorption band at 1682 cm^{-1} in the IR spectrum. The IR spectrum of **1** also showed an absorption band due to a hydroxyl group (3410 cm^{-1}). The characteristic proton and carbon signals of **1** were assigned by HMQC analysis of **1** (Table 1). The two partial structures of **1** were figured out by interpretation of the COSY and HMBC spectra as shown in Figure 1, but the adjacency of the two partial structures was not defined by spectroscopic analysis. The total structure of labuanine A was finally determined as 10,11-dihydro-8-hydroxybenzo[*b*]pyrido[4,3,2-*de*][1,10]phenanthrolin-9(9*H*)-one (**1**) on the basis of the chemical conversion from **1** to the known compound **2** by air oxidation[10] in DMSO. Compounds **2**, **3**, and **4** were respectively identified as 9-hydroxybenzo[*b*]pyrido[4,3,2-*de*][1,10]phenanthrolin-8(8*H*)-one,[8] 9-aminobenzo[*b*]pyrido[4,3,2-*de*][1,10]phenanthrolin-8(8*H*)-one[9] and biemnadin[10] by comparison of the physical data (FABMS and ^{1}H and ^{13}C NMR spectra) with previously reported data. Compound **2** was a synthetic regioisomer[8] of a marine pyridoacridine alkaloid, meridine,[11] which was isolated from the ascidian *Amphicarpa meridiana*. Compound **3** was also a synthetic regioisomer[9] of cystodamine,[12] which was isolated from the Mediterranean ascidian *Cystodytes delle chiajei*. Furthermore, the structure of cystodamine was later revised to be 11-hydroxyascididemin.[13] It is noteworthy that compounds **2** and **3** were the first case of isolation from a natural source and compound **2** might be an artefact metabolite produced from labuanine A (**1**) by air oxidation.

Table 1. ^{1}H and ^{13}C NMR data for labuanine A (**1**) (500 MHz and 125 MHz in DMSO-d_6)

No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))
1	131.0	8.41 (d, 7.9)
2	132.9	8.18 (dd, 7.9, 7.3)
3	131.5	8.11 (dd, 7.9, 7.3)
4	124.5	9.09 (d, 7.9)
4a	122.8	
4b	136.8	
5	121.2	9.18 (d, 5.5)
6	149.4	9.30 (d, 5.5)
7a	142.6–142.4	
8	157.8–157.6	
8a	99.0	
9	193.5	
10	34.5	2.93 (t, 7.6)
11	40.5	4.11 (t, 7.6)
12a	157.8–157.6	
12b	142.6–142.4	
12c	116.2	
13a	144.0	

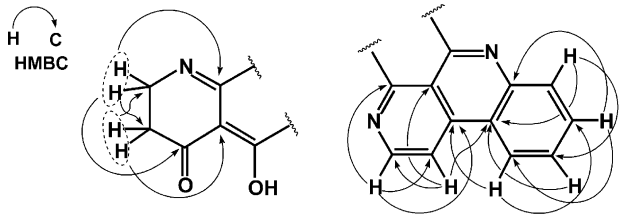

Figure 1. Partial structures of labuanine A (**1**) with key HMBC correlations.

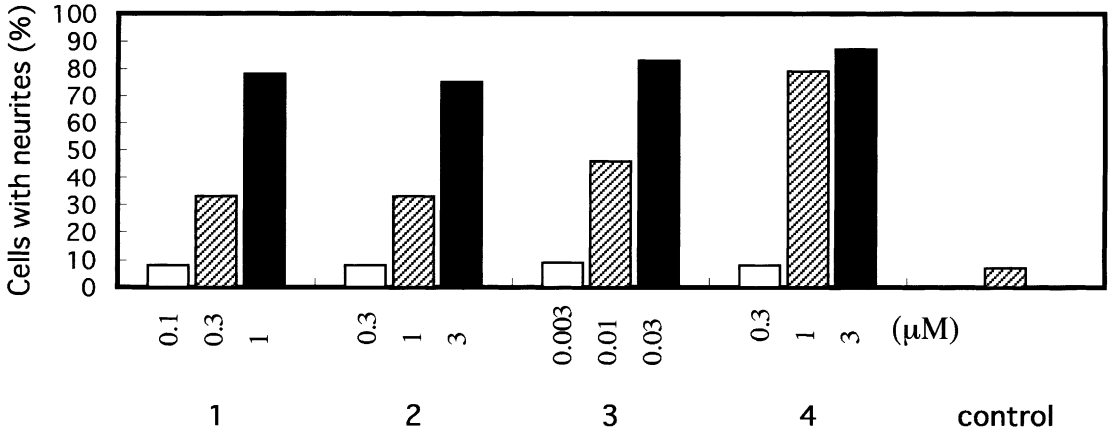

Figure 2. Quantification of neurites induced by compounds **1**–**4**. After 48 h incubation with samples, morphological changes in Neuro 2A cells were observed under microscope. The cells which have longer processes than the diameter of the cell body were evaluated as neurite-bearing cells.

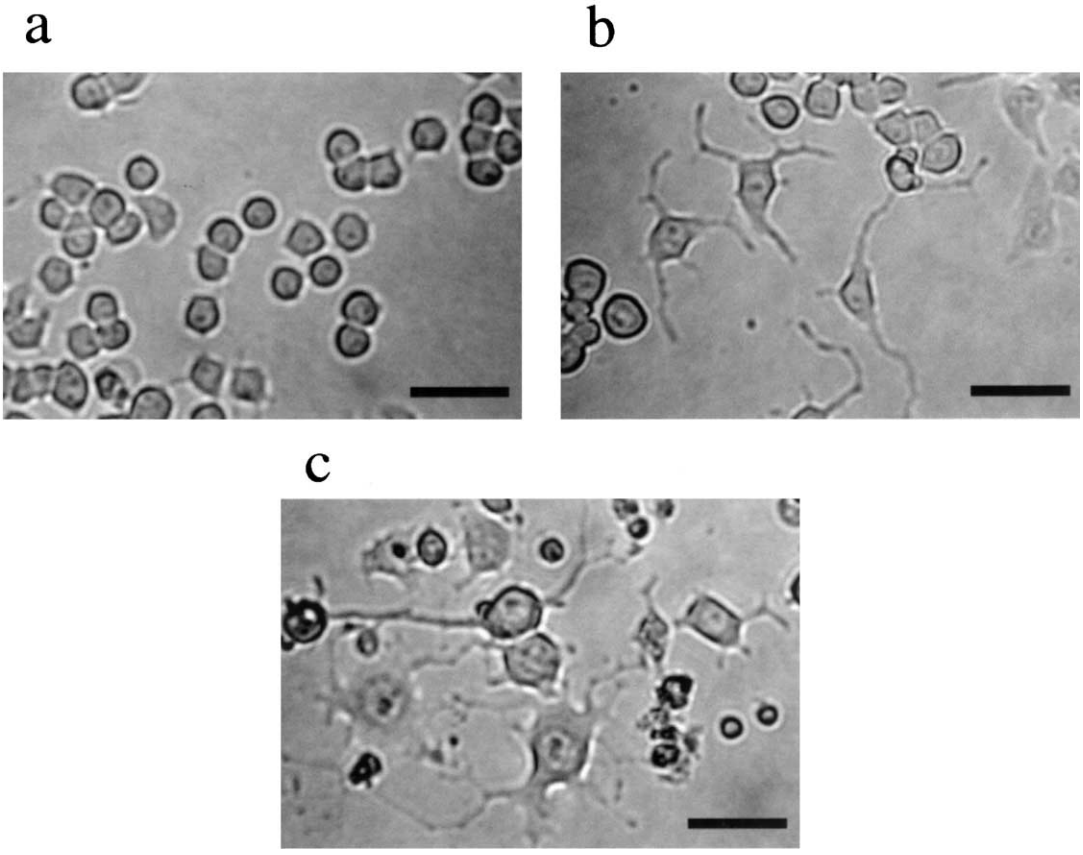

Figure 3. Photomicrographs of compound **3** or etoposide-induced morphological changes in Neuro 2A cells. Cells were cultured in the absence (control; a) or presence of compound **3** (0.03 μM; b) or etoposide (0.6 μM; c) for 72 h (Bar = 100 μm).

The data for the neurite outgrowth activity of these pyridoacridine alkaloids is shown in Figure 2. Compound **3** showed remarkable neuritogenic activity among them. More than 50% of the cells put out neurites by 72 h treatment with 0.01 μM concentration of **3**. However, at higher concentration than 0.3 μM, **3** was cytotoxic. Compounds **1**, **2** and **4** induced neurite outgrowth in more than 50% of cells at 1–3 μM concentration. The wide difference in neuritogenic activity between **2** and **3** implies the importance of the amino group at C-9 in **3** for neuritogenic activity. The morphological change with neurite outgrowth in Neuro 2A cells is generally classified to multipolar, unipolar, and bipolar types.[14] Compounds **1**–**4** induced multipolar type of neurite outgrowth in Neuro 2A (Fig. 3). The morphological change by the treatment with **3** was associated with an increase of acetylcholinesterase (AChE) activity,[15] which is a marker of functional neuronal differentiation in Neuro 2A cells. Compound **3** elicited 4-fold AChE activity after 48 h treatment at 0.03 μM concentration in comparison with the control (Fig. 4). This result suggested that **3** induced neuronal differentiation against Neuro 2A cells not only morphologically but also functionally. On the other hand, these compounds did not induce neurite outgrowth in rat pheochromocytoma PC12 cells.

Neuronal differentiation closely relates to the cell cycle. We investigated the effect of **3** on the cell cycle of Neuro 2A cells. For cell cycle analysis, Neuro 2A cells grown by random culture were used, and flow cytometric analysis was carried out at 24 h after addition of the drug. The treatment of Neuro 2A cells with 0.03 μM concentration of **3** remarkably increased the percentage of cells in the G2/M phase compared with that of the control cells (Fig. 5).

Neuronal differentiation by an endogenous differentiation inducer such as NGF was well investigated and the essential signal cascades for differentiation were eluci-

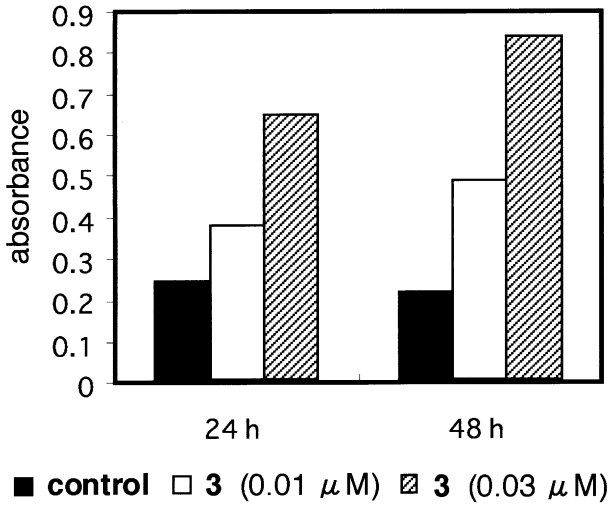

Figure 4. Effect of compound **3** on AChE amount in Neuro 2A cells.

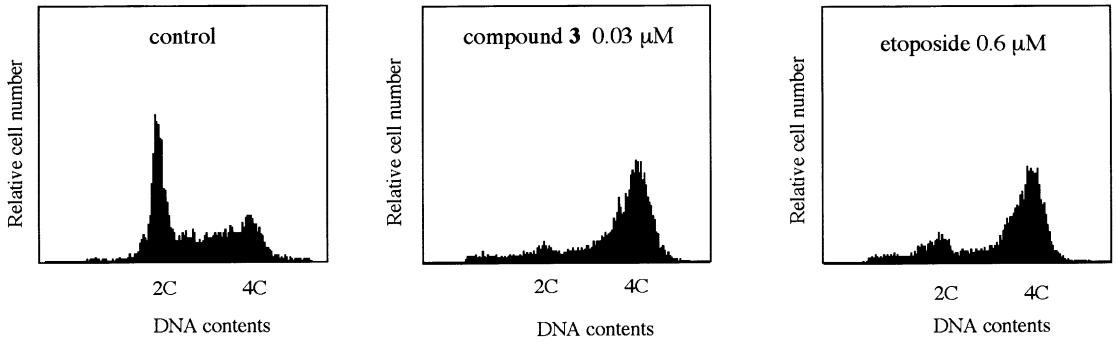

Figure 5. The action of compound **3** and etoposide on the cell cycle of Neuro 2A cells. Neuro 2A cells were exposed to compound **3** or etoposide for 24 h, and cell-cycle distribution was determined by flow cytometric analysis.

dated. NGF arrests the cell cycle at the G1 phase[16] and induces neuronal differentiation via Ras/Raf/MEK/ERK signaling pathway after cell cycle arrest.[17] On the other hand, the action mechanism of the exogenous differentiation inducers having low molecular weight was poorly investigated, although various types of compounds were reported to induce differentiation against various neuronal cell lines. Another pathway, which is activated by a low molecular differentiation inducer and not mediated by Ras, has been defined.[18] Compound **3** may induce neuronal differentiation through a different mechanism from that of the endogenous inducers, since **3** does not arrest cell cycle at the G1 phase even after 48 h treatment. The pyridoacridine alkaloids isolated from ascidians, marine sponges, and anemones, exhibit interesting properties.[19] It was elucidated that several marine pyridoacridine alkaloids intercalate into DNA and cleave the DNA double helix or inhibit the action of topoisomerase II (TOPO II).[20] TOPO II catalyses topological genomic changes essential for many DNA metabolic processes. Etoposide, a potent TOPO II inhibitor, also induced multipolar neuritogenesis and arrested cell cycle at the G2/M phase against Neuro 2A cells the same as pyridoacridine alkaloids **1**–**4**. This finding indicates that the neuronal differentiation by these pyridoacridine alkaloids in Neuro 2A may relate with inhibition of topoisomerase II. More detailed biochemical study is needed for understanding the action mechanism of the differentiation in neuroblastoma by pyridoacridine alkaloids.

Experimental

Isolation of pyridoacridine alkaloids 1–4 from the marine sponge *Biemna fortis*

The titled sponge (2.35 kg, dry weight) collected in August, 2001 at Labuanbajo, West Flores, Nusa Tenggara Timur, Indonesia, was extracted with MeOH (5 L) at room temperature three times and the solvent was removed by evaporation under reduced pressure to obtain a MeOH extract (87 g). The MeOH extract (87 g) was partitioned into an EtOAc–5% aq HCl mixture, and the 5% aq HCl-soluble portion was further partitioned into an EtOAc–water mixture after neutralization with aq NaOH. The EtOAc portion was dried over Na_2SO_4 and evaporated to obtain an alkaloid fraction (Fr. AK, 240 mg). Fr. AK (240 mg) was separated by reversed phase HPLC (Mightysil RP-18 GP, $CH_3CN/H_2O = 40:60$, 0.1% TFA) to give five fractions (Fr. AK1–Fr. AK5). Active Fr. AK-3 (9 mg) was further purified by reversed-phase HPLC (Mightysil RP-18 GP, $CH_3CN/H_2O = 25:75$, 0.1% TFA) to furnish new pyridoacridine alkaloid, labuanine A (**1**) (6 mg, 0.007% from MeOH extract) as a TFA salt. Fr. AK-2 (31 mg) was further separated by reversed phase HPLC (Mightysil RP-18 GP, $CH_3CN/H_2O = 22:78$, 0.1% TFA) to furnish compounds **2** (6 mg, 0.007%) and **3** (13 mg, 0.014%) as TFA salts. Fr. AK-4 (83 mg) was also further separated by reversed-phase HPLC (Mightysil RP-18 GP, $CH_3CN/H_2O = 33:67$, 0.1% TFA) to furnish compound **4** (16 mg, 0.018%) as a TFA salt. The TFA salts of these alkaloids were once dissolved in water and neutralized with 0.001 N aq NaOH and then extracted with $CHCl_3$ to give free alkaloids.

Labuanine A (1). Yellow amorphous solid, IR V_{max}(KBr) cm^{-1}: 3410, 2360, 1682, 1210. FABMS m/z 302 $(M+H)^+$. HR-FABMS m/z calcd for $C_{18}H_{12}N_3O_2$: 302.0929, found 302.0904. UV λ_{max} (MeOH) nm (ε): 357 (6067), 317 (5190), 288 (7720), 227 (18,000). ^1H and ^{13}C NMR (TFA salt): as shown in Table 1.

2. Yellow amorphous solid, FABMS m/z 322 $(M+Na)^+$. HR-FABMS m/z calcd for $C_{18}H_9N_3O_2Na$: 322.0593, found 322.0587. UV λ_{max} (MeOH) nm (ε): 365 (5270), 277 (10,600), 227 (15,000). ^1H NMR (500 MHz, $CDCl_3$) δ 9.31 (1H, d, $J = 5.5$ Hz, C_6-H), 8.91 (1H, d, $J = 5.5$ Hz, C_{11}-H), 8.70 (1H, d, $J = 5.5$ Hz, C_5-H), 8.62 (1H, d, $J = 7.9$ Hz, C_4-H), 8.61 (1H, d, $J = 7.9$ Hz, C_1-H), 7.96 (1H, dd, $J = 7.9$, 7.3 Hz, C_2-H), 7.85 (1H, dd, $J = 7.9$, 7.3 Hz, C_3-H), 7.13 (1H, d, $J = 5.5$ Hz, C_{10}-H). ^1H NMR (500 MHz, CF_3COOD) δ 9.53 (1H, d, $J = 6.1$ Hz), 9.35 (1H, d, $J = 6.1$ Hz), 8.91 (1H, d, $J = 7.9$ Hz), 8.89 (1H, d, $J = 7.0$ Hz), 8.54 (1H, d, $J = 7.9$ Hz), 8.29 (1H, t, $J = 7.9$ Hz), 8.18 (1H, t, $J = 7.9$ Hz), 7.64 (1H, d, $J = 7.0$ Hz). ^{13}C NMR (125 MHz, CF_3COOD) δ 179.0, 175.0, 147.3, 147.2, 146.6, 145.2, 141.8, 139.4, 137.8, 137.3, 135.4, 133.9, 126.9, 126.0, 121.4, 118.4, 117.7, 114.2.

3. Yellow amorphous solid, FABMS m/z 299 $(M+H)^+$. HR-FABMS m/z calcd for $C_{18}H_{11}N_4O$: 299.0933, found 299.0951. UV λ_{max} (MeOH) nm (ε): 373 (7020), 282

(14,100), 231 (20,000). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.25 (1H, d, J = 5.5 Hz, C_6-H), 9.03 (1H, d, J = 5.5 Hz, C_5-H), 8.94 (1H, d, J = 7.9 Hz, C_4-H), 8.42 (1H, d, J = 6.1 Hz, C_{11}-H), 8.35 (1H, d, J = 7.9 Hz, C_1-H), 8.01 (1H, dd, J = 7.9, 7.3 Hz, C_2-H), 7.90 (1H, dd, J = 7.9, 7.3 Hz, C_3-H), 6.95 (1H, d, J = 6.1 Hz, C_{10}-H), 7.95 (1H, br, –NH_2), 8.94 (1H, br, –NH_2). The ^{1}H NMR spectrum of **3** was directly identified with that[9] of the authentic sample.

4. Yellow amorphous solid, FABMS m/z 430 $(M+H)^+$. ^{1}H NMR (500 MHz, TFA salt in DMSO-d_6) δ 9.17 (1H, d, J = 5.5 Hz, C_6-H), 8.95 (1H, d, J = 5.5 Hz, C_5-H), 8.81 (1H, d, J = 7.9 Hz, C_4-H), 7.85 (1H, dd, J = 7.9, 7.3 Hz, C_2-H), 7.76 (1H, dd, J = 7.9, 7.3 Hz, C_3-H), 7.74 (1H, d, J = 7.3 Hz, C_{14}-H), 7.69 (1H, d, J = 7.9 Hz, C_1-H), 7.10 (1H, t, J = 7.3 Hz, C_{16}-H), 6.62 (1H, t, J = 7.3 Hz, C_{15}-H), 6.35 (1H, d, J = 7.3 Hz, C_{17}-H), 3.95 (1H, m, C_9-H), 3.71 (1H, m, C_{12}-H), 3.64 (1H, m, C_9-H), 3.58 (1H, dd, J = 12.8, 13.4 Hz, C_{12}-H), 3.27 (1H, dd, J = 12.8, 4.9 Hz, C_{12a}-H), 2.99 (1H, m, C_{10}-H), 2.91 (1H, m, C_{10}-H), 7.41 (1H, s, N_{18}-H), 10.30 (1H, br, N_8-H). ^{13}C NMR (125 MHz, TFA-salt in DMSO-d_6) δ 190.9 (C_{13}), 162.7 (C_{10a}), 155.1 (C_{18b}), 153.9 (C_{7b}), 148.7 (C_6), 145.3 (C_{17a}), 144.3 (C_{7a}), 143.2 (C_{19a}), 137.5 (C_{4b}), 134.9 (C_{16}), 132.1 (C_2), 129.3 (C_1), 128.5 (C_3), 125.6 (C_{14}), 124.1 (C_4), 121.2 (C_{4a}), 120.2 (C_5), 117.6 ($C_{13a,}$ C_{15}), 115.8 (C_{17}), 114.5 (C_{4c}), 95.3 (C_{10b}), 54.4 (C_{18a}), 44.6 (C_{12a}), 40.5 (C_{12}), 38.2 (C_9), 26.5 (C_{10}).

Chemical conversion from 1 to 2

Air was bubbled into a solution of compound **1** (0.8 mg) in DMSO (1 mL), and the mixture was stirred at 60 °C for 48 h. After removal of solvent by lyophilization, crude product was purified by reversed phase HPLC (Mightysil RP-18 GP, CH_3CN/H_2O = 22:78, 0.1% TFA) to furnish a product (0.5mg), which was identified with compound **2** by HPLC, FABMS, and ^{1}H NMR (as free alkaloid).

Neurite outgrowth assays

The cells were plated on 24-well plates at a density of 1×10^4 cells per well with 1 mL of the culture medium. After 24 h cultivation, the medium was exchanged for fresh medium, and a testing sample as 10 μL of an ethanol solution was added to each well. After 72 h incubation, morphological changes in the cells were observed under phase contrast microscope. The cells, which have processes longer than the diameter of the cell body, were evaluated as neurite-bearing cells. The percentage of the cells with neurites in a particular culture was determined by counting 300 cells at least in the photomicrographs of the areas where the cell density was representative.

Assay for acetylcholinesterase activity

The production of acetylcholinesterase (AChE) per 5×10^4 cells was measured after 24 and 48 h treatment of sample. The activity was evaluated by measuring the rate of hydrolysis of acetylthiocholine colorimetrically as described in the previous report.[14]

Cell-cycle analysis

The cell suspension of Neuro 2A cells (4×10^4 cells in 1 mL of the culture medium) was plated on 24-well plate and incubated for 24 h. After medium exchange, an ethanol solution (10 μL) of the testing sample was added and further incubated for 24 h. The culture medium of the cell suspension was removed by centrifugation (1000g for 3 min). The collected cells were dyed by DNA-Prep Reagents Kit for 20 min. Then, the supernatant was removed by centrifugation (500g for 5 min) and the resulting cell suspension in 500 μL of D-PBS (−) solution was filtered by 40-μm nylon mesh filter. The cell-cycle analysis of the filtrate was carried out on a FACSCalibur (Becton Dickinson, λ_{ex} = 493 nm, λ_{em} = 630 nm).

Acknowledgements

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