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Dysideamine, a new sesquiterpene aminoquinone, protects hippocampal neuronal cells against iodoacetic acid-induced cell death

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ABSTRACT

In the course of our search for neuroprotective agents, dysideamine (**1**), a new sesquiterpene aminoquinone, was isolated along with bolinaquinone (**2**) from Indonesian marine sponge of *Dysidea* sp. Compounds **1** and **2** showed neuroprotective effect against iodoacetic acid (IAA)-induced cell death at 10 μM concentration in mouse HT22 hippocampal neuronal cells. Dysideamine (**1**) inhibited production of reactive oxygen species (ROS) by IAA treatment, whereas it exhibited no effect on depletion of intracellular ATP of the IAA-treated HT22 cells. Moreover, **1** induced neurite outgrowth against mouse neuroblastma Neuro 2A cells with increase of acetylcholinesterase (AChE) activity, which is a marker of neuronal differentiation.

1. Introduction

Ischemic stroke is the leading cause of adult disability and death. Approximately six million people died by stroke in 2005. Ischemic stroke occurs when normal blood supply to brain was disrupted due to artery blockage by a blood clot. Neuronal cell death is induced by depriving oxygen and nutrients. On the other hand, IAA is a well known irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is one of the enzymes on the glycolysis pathway. It was reported that the IAA-treated neuronal cells induced cell death following depletion of intracellular ATP, mitochondrial dysfunction, and production of reactive oxygen species (ROS).[1–4] These observations are similar with those of in vivo ischemic stroke. Then, IAA-induced cell death is used as a model of ischemic stroke. In addition, the recent studies indicated that ROS might be major trigger leading cell death in ischemic stroke and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases by activating various cellular signaling pathways including mitogen-activated protein kinase (MAPK) pathway.[5–7] Therefore, the compounds exhibiting neuroprotective effect against ROS are expected to become a new drug for these diseases. In fact, edaravone, which prevented oxidative injury, has been approved for the treatment of acute cerebral infarction.[8]

On the other hand, the neurotrophic factors such as nerve growth factor (NGF) play important roles in growth, differentiation, and survival in neuronal cells.[9] Therefore, they are expected to have a regenerative activity in the injured tissues in ischemic stroke or neurodegenerative diseases. However, because of their inability to pass the blood–brain barrier and vulnerability to hydrolytic enzymes, they are not able to use for medical treatment.[10,11] Then, the substances inducing differentiation against neuronal cells are also screened in the drug development for ischemic stroke and neurodegenerative diseases.[12,13] The several compounds such as KT7515 and T-817MA, are under clinical study.[14,15] Based on these findings, the compounds, which have both of the protective activity from oxidative stress and the activity inducing neuronal differentiation, would be a new lead for drug candidate.

Previously, we have isolated crambescidin 800, a pentacyclic guanidine alkaloid, as neuronal protective substance against glutamate-induced oxidative stress.[16] In the course of our search for neuroprotective substances, we isolated dysideamine (**1**), a new sesquiterpene aminoquinone, along with bolinaquinone (**2**) from Indonesian marine sponge of *Dysidea* sp. 05C33. Compounds **1** and **2** showed neuroprotective activity against IAA-induced cell death in mouse HT22 hippocampal neuronal cells. From the analysis for action-mechanism, **1** was found to inhibit production of ROS by IAA-treatment and also induce neurite outgrowth against mouse neuroblastma Neuro 2A cells. In this paper, the structure elucidation and neuroprotective effect of **1** are presented.

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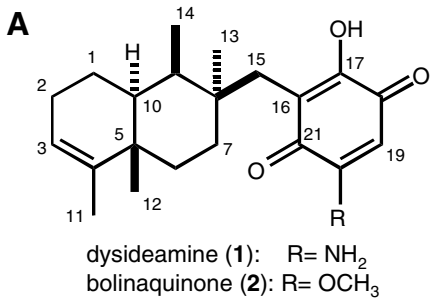

Figure 1. Chemical structures of active compounds (A) and picture of Indonesian marine sponge of *Dysidea* sp. 05C33 (B).

2. Results and discussion

2.1. Isolation of dysideamine (1) and bolinaquinone (2)

The MeOH extract of the dried marine sponge of *Dysidea* sp. 05C33, which was collected in 2005 at Indonesia, showed neuroprotective activity against IAA-induced cell death in mouse HT22 hippocampal neuronal cells. On the guidance of bioassay, the MeOH extract was separated by solvent partition, SiO_2 column chromatography, and HPLC to afford an active compound named dysideamine (**1**). A related known compound, bolinaquinone (**2**), was also isolated from the 90% aq MeOH extract by the similar procedure (Fig. 1).

2.2. Structure elucidation of dysideamine (1)

Dysideamine (**1**) was obtained as a red purple amorphous solid. The ESI-TOF MS of **1** showed a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 366, and the molecular formula was determined as $C_{21}H_{29}NO_3Na$ by high-resolution (HR-) ESI-TOF MS and NMR analysis. The UV absorption maxima (λ_{max}: 314 nm (ε 10,500), 205 nm (ε 14,200)) and the absorption band at 1583 cm^{-1} in the IR spectrum of **1** suggested that **1** has quinone moiety. The IR absorptions at 3329 and 3294 cm^{-1} also suggested the presence of amine and hydroxyl groups. The ^1H and ^{13}C NMR spectra of **1** showed the signals ascribable to two olefinic protons (δ_H 5.62, 5.15), four methyl protons (δ_H 1.54, 0.99, 0.96, 0.92), six olefinic carbons (δ_C 155.7, 150.4, 144.1, 120.7, 114.8, 95.9), and two keto carbonyl carbons (δ_C 183.4, 180.1). Four partial structures (A–D) in **1** were revealed by COSY and HMQC analysis (Fig. 2). And, the connectivity of these partial structures was figured out on the basis of the HMBC correlations as shown in Figure 2. All the proton- and carbon-signals were assigned as shown in Table 1, and the planar structure of **1** was elucidated as shown in Figure 2.

The relative stereostructure of **1** was revealed by the NOESY correlations between H-6, H-10 and H-13; H-7, H-12, H-15 and H-14; H-9 and H-10 as shown in Figure 3.

Bolinaquinone (**2**) was identified by ESI-TOF-MS and 2D NMR analysis and comparison with authentic spectral data.[17]

Dysideamine (**1**) and bolinaquinone (**2**) belong to sesquiterpene quinones having unique rearranged drimane skeleton. So far, many sesquiterpene quinones having C-9 quinone moiety (e.g., ilimaquinone[18] and isospongiaquinone[19]) were reported. Dysideamine (**1**) and bolinaquinone (**2**) are rare sesquiterpene quinones having C-8 quinone moiety.

2.3. Neuroprotective effect of dysideamine (1) and bolinaquinone (2)

The neuroprotective effects of **1** and **2** against IAA-induced cell death were examined by MTT method. As shown in Figure 4A, HT22 cells, which were treated with IAA for 2 h, induced cell death during additional 20 h incubation as described previously.[1] Dyside-

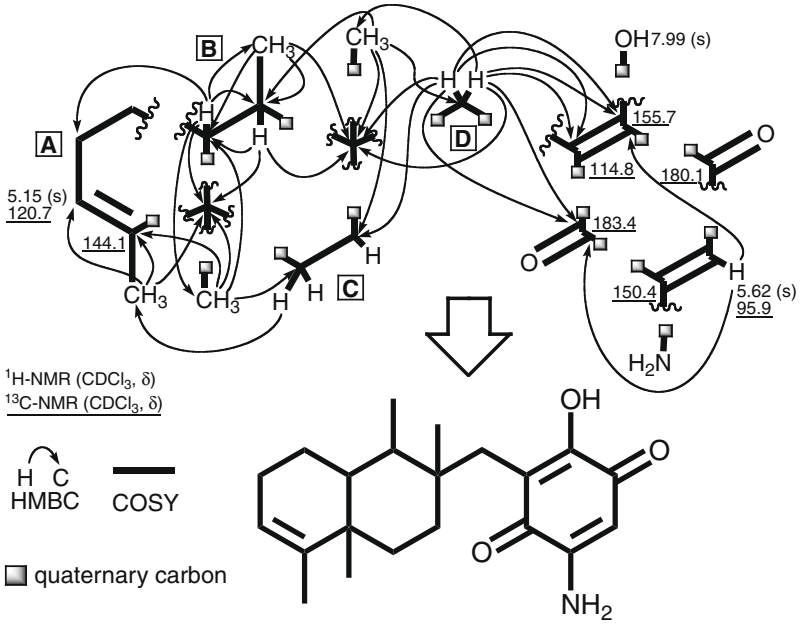

Figure 2. COSY and HMBC correlations for **1**.

Table 1
^{1}H and ^{13}C NMR data for **1**

Position	Dysideamine (**1**)	
	δ_C (ppm)	δ_H (ppm, *J* in Hz)
1	24.8	1.74 (m)
		1.20 (dt, 13.2, 3)
2	26.9	2.04 (br s)
3	120.7	5.15 (s)
4	144.1	
5	38.0	
6	32.2	1.44 (dt, 13.2, 3.6)
		1.27(dd, 13.8, 3.6)
7	28.9	1.70 (dd, 13.8, 3.6)
		0.99 (d, 7.4)
8	39.5	
9	45.1	1.47 (m)
10	40.9	1.89 (ddd, 12.6, 4.2, 1.8)
11	17.9	1.54 (s)
12	12.5	0.96 (s)
13	24.0	0.92 (s)
14	20.0	0.99 (d, 7.4)
15	34.1	2.28 (d, 12.6)
		2.52 (d, 12.6)
16	114.8	
17	155.7	
18	180.1	
19	95.9	5.62 (s)
20	150.4	
21	183.4	
22		
OH		7.99 (s)

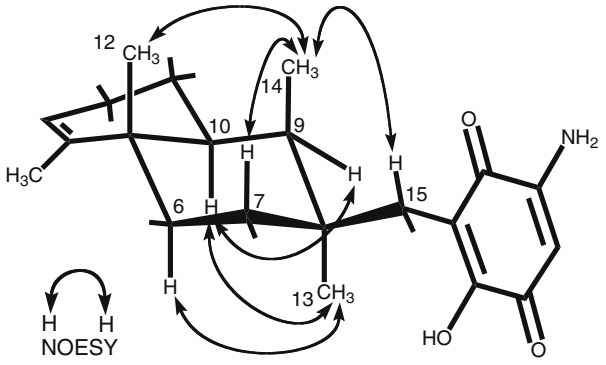

Figure 3. NOESY correlations for **1**.

amine (**1**) exhibited neuroprotective effect against IAA-induced cell death for dose-dependent fashion from 1.0 μM, and 43% of HT22 cells were survived, when 10 μM of **1** was added into the culture medium. Bolinaquinone (**2**) (10 μM) also showed neuroprotective effect (57% survival) against IAA-induced cell death (Fig. 4B).

2.4. Effect of dysideamine (1) on the depletion of intracellular ATP by IAA

The IAA-treated HT22 cells induce depletion of intracellular ATP, mitochondrial dysfunction, and increase of ROS production.[2–4] Then, we investigated the effect of **1** on the intracellular ATP and ROS production involved in the cell death by IAA. After 2 h incubation with IAA, the amount of intracellular ATP in HT22 cells was markedly decreased to less than 20% of those in non-treated HT22 cells (Fig. 5). Then, the cells recovered intracellular ATP content by 8 h from the IAA-treatment. Dysideamine (**1**) slightly hastened to recover intracellular ATP from 4 h to 8 h. However, no significant effect of **1** was observed in comparison with those for the IAA-treated cells without **1**.

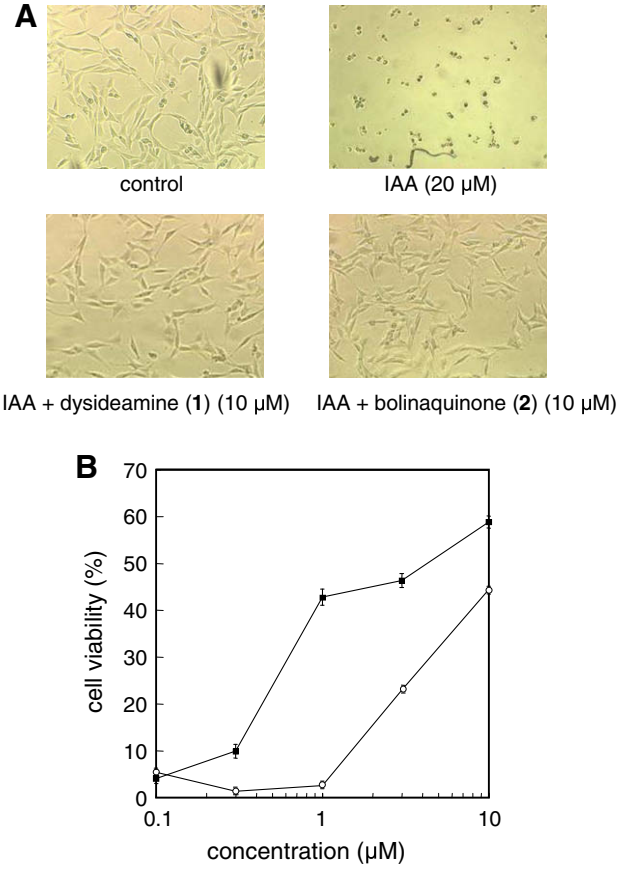

Figure 4. Neuroprotective effect of **1** and **2** against IAA-induced cell death. HT22 cells were treated with IAA for 2 h to induce cell death. Then, the medium in each well was replaced with normal growth DMEM, and **1** or **2** was added as 1.0 μl of EtOH solution. The plates were incubated for additional 20 h, and the cell proliferation was detected by MTT colorimetric assay. Results are the mean ± SEM values ($n = 3$). (A) The cell morphology of the IAA-treated HT22 cells in the absence or presence of **1** or **2**. (B) Dose-dependent protection by **1** (open circle) and **2** (closed square) was shown.

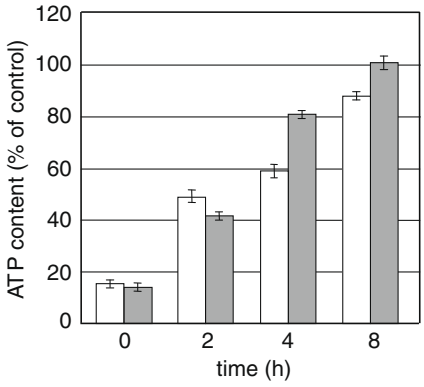

Figure 5. Effect of **1** on the IAA-induced depletion of intracellular ATP. The ATP level was measured by CellTiter-Gro Luminescent Cell Viability Assay Kit according to manufacture protocol. Results are the mean ± SEM values ($n = 3$). Open bar: absence of compound **1**, closed bar: presence of compound **1**.

2.5. Effect of dysideamine (1) on the production of reactive oxygen species

The effect of **1** on the production of ROS was investigated by using fluorescent dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The IAA-treated HT22 cells increased production of ROS, and the maximal production was observed at 2 h after IAA-treatment.

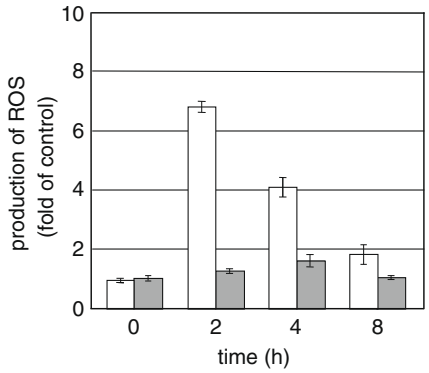

Figure 6. Effect of **1** on the IAA-induced intracellular ROS production. The intracellular ROS level was presented as the fold of the control. Results are the mean ± SEM values (n = 3). Open bar: absence of compound **1**, closed bar: presence of compound **1**.

And then, the intracellular ROS was decreased in time-dependent fashion. Dysideamine (**1**) significantly restrained production of ROS in the IAA-treated HT22 cells (Fig. 6). This data suggested that **1** could prevent IAA-induced cell death by inhibiting ROS production. This result also supported that ROS might be trigger leading the cell death in ischemic stroke and neurodegenerative diseases.[20–24]

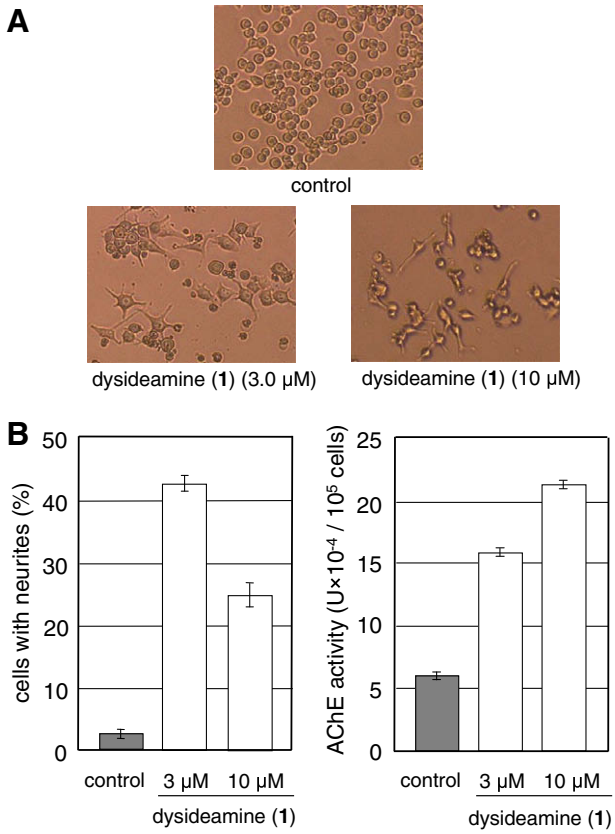

Figure 7. Quantification of neuritis induced by **1**. Neuro 2A cells (1×10^4 cells/ml/well) in the 24-well plates were treated with indicated concentration of **1** for 48 h. The morphological changes in the cells were observed under phase contrast microscope (A). The percentage of the cells with neurites in a particular culture was determined by counting 300 cells at least in the photomicrographs of the areas where the cell density was representative (B left side). The production of acetylcholinesterase (AChE) per 5×10^4 cells was measured after 48 h treatment of **1**. The activity was evaluated by measuring the rate of hydrolysis of acetylthiocholine colorimetrically (B right side).

2.6. Effect of dysideamine (1) on the induction of neurite outgrowth

The compounds inducing differentiation against neuronal cells are expected to become new lead for medical treatment of ischemic stroke and neurodegenerative diseases.[13,14] Then, we next examined whether dysideamine (**1**) has an activity inducing neurite outgrowth in mouse neuroblastoma Neuro 2A cells or not (Fig. 7). Compound **1** induced neurite outgrowth more than 40% of the cells treated with 3.0 μM concentration of **1**. Although 25% of the cells treated with 10 μM concentration of **1** were induced neurite outgrowth, cytotoxic effect was slightly observed at this concentration. Moreover, an increase of acetylcholinesterase (AChE) activity, which is a marker of functional neuronal differentiation in Neuro 2A cells, was associated with the morphological change by dysideamine (**1**) treatment (Fig. 7B). This result indicated that dysideamine (**1**) induced neuronal differentiation against Neuro 2A cells not only morphologically but also functionally.

Therefore, dysideamine (**1**) might be effective to both ischemic stroke and neurodegenerative diseases. The in vivo efficacy and detail of action-mechanism of neuroprotective activity of **1** are under way.

3. Experimental

3.1. Chemicals and reagents

Mouse HT22 hippocampal cell line was kindly provided from Dr. K. Ishige, (College of Pharmacy, Nihon University).[25–27] Mouse neuroblastoma Neuro 2A cell line was also gifted from Dr. A. Baba (Graduate School of Pharmaceutical Sciences, Osaka University). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was obtained from Tissue Culture Biologicals (Tulare, CA). Iodoacetic acid (IAA), 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and other reagents were purchased from Sigma Chemical Co., Ltd (St. Louis, MO).

3.2. Measurement of cell viability

HT22 cells were maintained in DMEM with 10% FBS. The assay of neuroprotective effect was carried out by Maher's method with minor modification.[1] A suspension of HT22 cells in DMEM containing 10% FBS was plated into 96-well plates (5×10^3 cells/well/100 μl) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h incubation, the medium was replaced with DMEM supplemented with 7.5% FBS, and the cells were treated with 20 μM iodoacetic acid (IAA) for 2 h. Then, the medium in each well was replaced with fresh DMEM containing 10% FBS, and the testing sample was added as 1.0 μl of EtOH solution. The plates were incubated for additional 20 h, and the cell proliferation was detected by MTT colorimetric assay.

3.3. Isolation of dysideamine (1) and bolinaquinone (2)

The dried marine sponge of *Dysidea* sp. 05C33 (300 g), which was collected in August, 2005 at Biak, Indonesia, was extracted with MeOH to obtain a MeOH extract (17.7 g). The MeOH extract (9.3 g), which exhibited neuroprotective activity (20% cell viability at 10 μg/ml) against IAA-induced cell death, was partitioned into a water–EtOAc mixture (1:1). The EtOAc soluble portion was further partitioned with *n*-hexane–90% aq MeOH mixture (1:1) to furnish a 90% MeOH extract (1.2 g) and *n*-hexane extract (1.9 g). The 90% MeOH extract (230 mg, 43% cell viability at 10 μg/ml) was fraction-

ated by SiO_2 gel column chromatography ($CHCl_3$:MeOH:H_2O) to give eleven fractions (Fr. M1–Fr. M11). The Fr. M6 (49 mg, 20% cell viability at 10 μg/ml) was then separated by HPLC (Cosmosil 5C18-AR, MeOH–H_2O = 95:5) to obtain an active fraction (Fr. M62 (20 mg)). The Fr. M62 (60% cell viability at 10 μg/ml) was further purified by HPLC (Cosmosil 5C18-AR, MeOH–H_2O = 8:2 containing 0.1% TFA) to afford **1** (13 mg). The Fr. M2 (4 mg, 60% cell viability at 10 μg/ml) was also purified by HPLC (Cosmosil 5C18-AR, MeOH–H_2O = 8:2) to furnish **2** (2 mg). Compound **2** was identified by mass and NMR analysis with the reported data for bolinaquinone.[17]

*Dysideamine (**1**)*: Red purple amorphous solid; $[\alpha]_D^{20}$ − 208 (*c* 0.3, $CHCl_3$); ESI-MS: *m/z* 366 $[M+Na]^+$. High resolution ESI-MS: Calcd for $C_{21}H_{29}NO_3Na$: *m/z* 366.2033. Found 366.2045. IR ν_{max} (KBr) cm^{-1}: 3329, 3294, 2922, 1583, 1381, 1215. UV λ_{max} (MeOH) nm (ε): 314 (10,500), 205 (14,200). ^1H NMR (600 MHz, $CDCl_3$, δ), ^{13}C NMR (150 MHz, $CDCl_3$, δ_c) spectra: as shown in Table 1.

3.4. Effect of dysideamine (1) on the IAA-induced production of ROS

HT22 cells were plated into 24-well plastic plates (5×10^4 cells/500 μl/well) and cultured for 24 h at 37 °C in normal growth medium. Then, the medium was replaced with DMEM supplemented with 7.5% FBS, and the cells were treated with 20 μM IAA for 2 h. Then, the medium in each well was replaced with normal growth medium, and **1** (10 μM) was added as 5.0 μl of EtOH solution. The cells were washed with serum-free medium after indicated time, and the harvested cells were treated with 10 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the serum free medium for 20 min under dark condition. Then, the cells were washed with serum free medium, and the fluorescence at 530 nm in response to excitation at 485 nm was measured.

3.5. Effect of dysideamine (1) on the IAA-induced depletion of intracellular ATP

HT22 cells (5×10^3 cells/100 μl/well) were treated with 10 μM of IAA for 2 h in the DMEM supplemented with 7.5% FBS. Then, the medium was replaced with normal growth medium, and the cells were incubated in the presence or absence of **1** for indicated time. The measurement of the intracellular ATP was performed by CellTiter-Gro Luminescent Cell Viability Assay Kit (Promega, Madison, WI) according to manufacture protocol.

3.6. Neurite outgrowth assays

Neuro 2A cells were maintained in DMEM with 10% FBS. 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 incubation, the medium was exchanged with fresh medium, and **1** as 10 μl of EtOH solution was added to each well. After 48 h incubation, the 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.

3.7. Assay for acetylcholinesterase activity

The production of acetylcholinesterase (AChE) per 5×10^4 cells was measured after 48 h treatment of **1**. The activity was evaluated by measuring the rate of hydrolysis of acetylthiocholine colorimetrically as described in the previous report.[28–30]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.04.025.

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