New Diphenazines with Neuronal Cell Protecting Activity, Phenazostatins A and B, Produced by *Streptomyces* sp.

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Phenazostatins A and B, new diphenazine compounds, were isolated from the culture broth of *Streptomyces* sp. 833 as new neuronal cell protecting substances which also showed free radical scavenging activity. In the cell assay, phenazostatins A and B inhibited glutamate toxicity in N18-RE-105 cells with EC_{50} values of 0.34 and 0.33 μM, respectively.

It has been reported that L-glutamate, a major neurotransmitter in the central nervous system, is extensively released during brain ischemia and induces subsequent neuronal cell death.[1,2] Recent studies indicate that oxygen radicals are produced through a variety of intracellular cascades in such events.[2] It was also reported that blockage of glutamate toxicity by free radical scavengers was effective to ameliorate brain ischemia injury.[3,4] Recently, some glutamate toxicity inhibitors of microbial origin such as carquinostatin A[5], lavanduquinocin[6], and aestivophoenins A and B[7] have been reported. In the course of our screening for free radical scavengers or inhibitors of glutamate toxicity using the neuronal hybridoma N18-RE-105 cells to prevent the brain ischemia injury, we previously isolated benzastatins A~G[8~10]. Further investigation have been in isolation of unique diphenazine compounds, phenazostatins A (**1**) and B (**2**) (Fig. 1). In addition, two simple phenazines, methyl ester of 6-acetylphenazine-1-carboxylic acid (**3**) and methyl saphenate[11] (**4**), were also detected in the same culture broth.

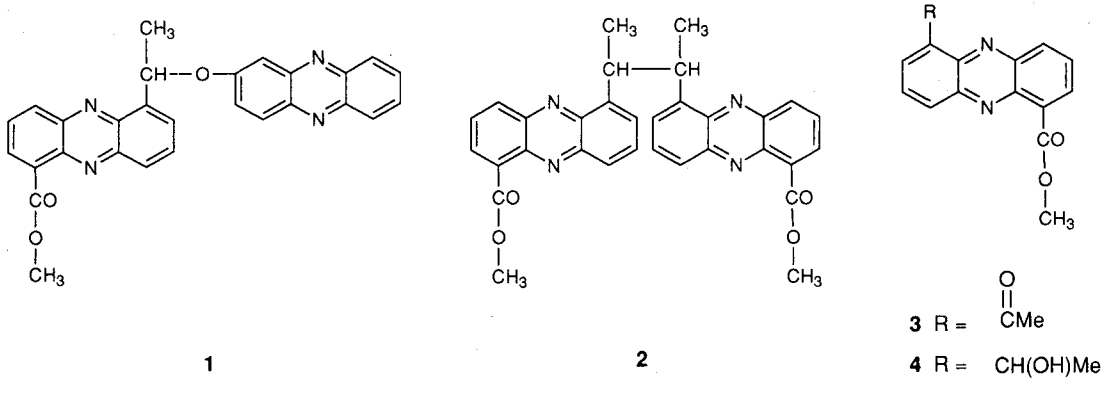
We described structure determination of **1** and **2** in separate paper[12]. We report here the taxonomy of the producing strain, fermentation, isolation, physicochemical properties and biological activities of **1**~**4**.

Materials and Methods

Taxonomy

Strain 833 was isolated from a soil sample collected in Jechun-city, Chungcheongbook-do, Korea. The taxonomic studies were carried out as described by the International *Streptomyces* Project (ISP).[13] For the evaluation of cultural characteristics, the strain was incubated in ISP media (Difco, U.S.A.) for 14~21 days at 28°C. The substrate and aerial mass color were assigned by Guide to Color Standard (Nihon, Shikisai Co., Ltd.). Physiological properties including utilization

Fig. 1. Structures of phenazostatins A (**1**), B (**2**) and related co-metabolites.



of carbon sources were examined by the method of PRIDHAM and GOTTLIEB.[14] The type of diaminopimelic acid (DAP) isomers in the cell wall was analyzed by the methods of BECKER *et al.*[15]

Media and Fermentation

A loopful of strain 833 from a mature slant culture was inoculated into a 500 ml Erlenmeyer flask containing 80 ml of sterile seed medium consisting of soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization) and cultured on a rotary shaker (150 rpm) at 28°C for 2 days. For the production of phenazostatins, 3 ml of the seed culture was transferred into one-liter Erlenmeyer flasks containing 150 ml of the above medium, and cultivated for 6 days using the same conditions. For the study on the time course of production of phenazostatins, each time 10 ml samples of the fermentation broth were drawn. After centrifugation at 3000 rpm for 10 minutes, the pH value, the packed cell volume (PCV), and the production of phenazostatins were measured. The antibiotics in the broth supernatants were extracted with EtOAc and their concentrations were measured by reserved phase HPLC (Cosmosil 4.6 × 150 mm) with a solution of MeOH - H_2O (85 : 15) at the UV absorption of 365 nm.

Inhibitory Activity against Glutamate Toxicity in N18-RE-105 Cells

N18-RE-105 cells[16] (mouse neuroblastoma clone N18TG-2 × Fisher rat 18-day embryonic neural retina) were maintained at 37°C in 25 cm^2 tissue culture flasks in 90% DMEM containing HAT (thymidine 0.14 mM, aminopterin 40 μM, hypoxanthine 0.1 mM) and 10% fetal calf serum under a humidified atmosphere of 5% CO_2, 95% air. Cells were plated in 96 well microplates at a density of 20,000 cells per well with 100 μl media. After culturing for 24 hours, the medium was removed and replaced with a medium containing 10 mM L-glutamate and/or drugs. Cytotoxicity was quantified after treatment for 24 hours by the measurement of the cytosolic enzyme, lactate dehydrogenase (LDH), which was released into the culture medium from degenerating cells. LDH activity was measured using commercial kit purchased from Promega. The percentage of cell death was calculated from the following formula: % cell death = A/(A + B)100, in which A and B are LDH activity in the culture media (supernatant) and in the cell lysates, respectively. EC_{50} value is the drug concentration necessary to reduce glutamate-induced cell death by 50%.[17]

Inhibitory Activity against Lipid Peroxidation in Rat Liver Microsomes

According to the method of OHKAWA *et al.*[18], rat liver microsomes were prepared and suspended in 100 mM Tris-HCl buffer (pH 7.4). Lipid peroxidation was initiated by adding 500 μM $FeSO_4 \cdot H_2O$. After 30 minutes at 37°C, the reaction was stopped by adding 3 M trichloroacetic acid in 2.5 N HCl. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive products. Percent inhibition was calculated as follows: $(1-(T-B)/(C-B)) \times 100$ (%), in which T, C, and B are absorbance values at 530 nm of the drug treatment, the control (peroxidation without a drug) and the 0 time control (no peroxidation), respectively.

Results

Taxonomic Studies of the Producing Strain

The strain 833 was cultured in various ISP media and the characteristics are summarized in Table 1. The substrate mycelia grew abundantly on yeast-extract - malt extract agar, oatmeal agar, and inorganic salts-starch agar, but grew poorly on glycerol-asparagine agar, tyrosine agar, and glucose-asparagine agar. The aerial mycelia grew abundantly on yeast-extract - malt extract agar and inorganic salts-starch agar, but didn't grow on peptone-yeast extraction iron agar. The aerial mass color was white or grayish white. Sclerotium, sporangium, and zoospores were not observed. The spore chains were observed to be *retinaculiaperti* type and each had more than ten spores per chain. The spores were cylindrical in shape, were $0.6 \sim 0.7 \times 0.8 \sim 0.9$ μm in size and had a smooth surface (Fig. 2). The isomer of DAP in whole cell hydrolysates of strain 833 was determined to be the LL-form. The physiological characteristics and the utilization of carbohydrates are shown in Table 2. Melanoid and soluble pigments were not observed. The strain utilized D-glucose, inositol, D-mannitol, raffinose, D-xylose, cellobiose, and D-galactose, but didn't utilize D-fructose, cellulose, L-arabinose, L-rhamnose, and sucrose. Based on the taxonomic properties described above, strain 833 was determined to belong to the genus *Streptomyces*.[19,20] The strain was deposited in Korean Collection for Type Culture, Korea Research Institute of Bioscience and Biotechnology, Korea, under the accession number KCTC-8796P.

Table 1. Cultural characteristics of strain 833.

Medium	Growth	Aerial mycelium	Substrate mycelium	Reverse color	Soluble pigment
Yeast extract-malt extract agar (ISP medium 2)	Good	Abundant, grayish white	Pale yellow	Brown	None
Oatmeal agar (ISP medium 3)	Good	Moderate, white	Pale yellow	Light brown	None
Inorganic salts-starch agar (ISP medium 4)	Good	Abundant, white	Pale brown	Brown	None
Glycerol-asparagine agar (ISP medium 5)	Poor	Poor, grayish white	Yellowish brown	Light brown	None
Peptone-yeast extract-iron agar (ISP medium 6)	Moderate	None	Pale yellow	Yellowish brown	None
Tyrosine agar (ISP medium 7)	Poor	Poor, grayish white	Pale brown	Brown	None
Glucose-asparagine agar	Poor	Poor, grayish white	Pale brown	Brown	None
BENNET's agar	Good	Abundant, whitish gray	Brown	Dark brown	None

Fig. 2. Scanning electron micrograph of spore chains of strain 833 on ISP-2 agar incubated at 27°C for 2 weeks (×20,000).

Bar represents 1 μm.

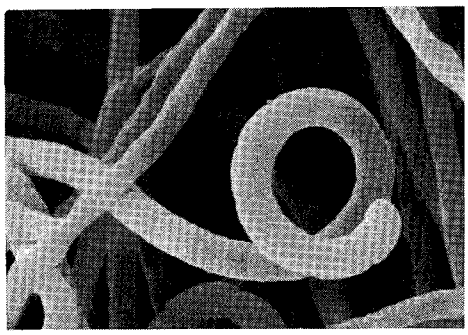


Table 2. Taxonomical characteristics of strain 833.

DAP type	LL
Spore chain	*Retinaculiaperti*
Spore surface	Smooth
Aerial mass color	Grayish white
Soluble pigments	None
Melanoid pigments	None
Growth temperature (°C)	10 - 34
Optimum temperature (°C)	25 - 28
Starch hydrolysis	Positive
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Milk peptonization	Negative
Hydrolysis of skim milk	Positive
Carbon utilization[a]	
D-Glucose	+
D-Fructose	-
Cellulose	-
Inositol	+
D-Mannitol	+
Raffinose	+
D-Xylose	+
L-Arabinose	-
Cellobiose	+
D-Galactose	+
L-Rhamnose	-
Sucrose	-

[a] +, Positive utilization; -, no utilization.

Fermentation

Fig. 3 presents the data for a typical fermentation in one-liter Erlenmeyer flask and give information regarding the production of **1**～**4**, pH, and packed cell volume. The production of **2**～**4** began at day 1 and reached maximun at around day 4 with yield of 2.8, 6.7, and 8.3 μg/ml, respectively. Among them, the production of **3** began to decrease drastically after 4 days of cultivation. On the other hand, the production of **1** began at day 4 and reached a maximum (0.41 μg/ml) at day 6.

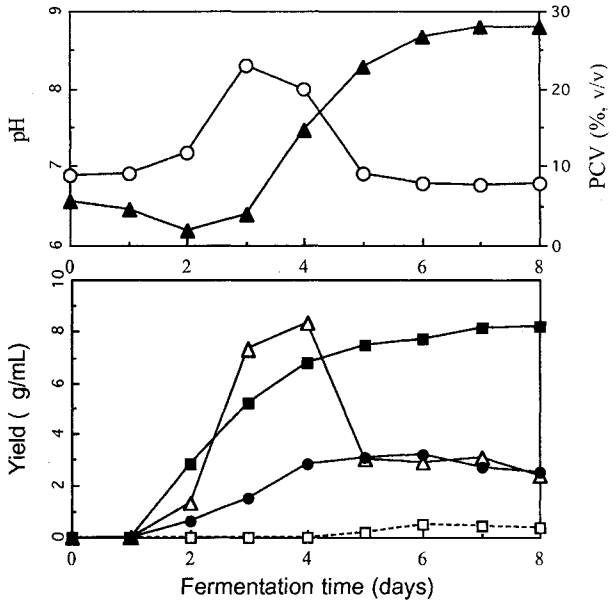
Isolation

The procedure for isolation of phenazostatins A (**1**), B (**2**) and related co-metabolites is shown in Scheme 1. The culture supernatant obtained from the culture broth (2 liters) was extracted with an equal volume of hexane three times and the hexane layer was concentrated *in vacuo*. The crude extract was charged to a silica gel (Merck art No 7734.9025) column followed by elution with hexane-EtOAc (4:1). Three active fractions containing **3** (fraction I), **1** and **2** (fraction II), and **4** (fraction III) were successively eluted. The fraction II was concentrated *in vacuo* and applied to a Sephadex

LH-20 column, which was developed with methanol. The active eluate was further purified by C-18 (YMC-gel ODS-A Lot No. 51252) column chromatography. The column was eluted with 80% aqueous MeOH to give two active fractions. These fractions were separately recrystallized in MeOH to afford **1** (0.8 mg) and **2** (8.5 mg) as yellow crystals.

Fig. 3. Time course production of phenazostatins A (**1**), B (**2**) and related co-metabolites.

pH (▲), PCV (○), **1** (□), **2** (●), **3** (△), **4** (■).



Physico-chemical Properties

Physico-chemical properties of **1** and **2** are summerized in Table 3. **1** and **2** are soluble in chloroform, ethyl acetate, acetone and dimethyl sulfoxide, slightly soluble in methanol and acetonitrile, and insoluble in water and *n*-hexane. After TLC on silica gel 60 F_{254} (Merck) with *n*-hexane - EtOAC (3 : 1), **1** and **2** showed Rf values of 0.23 and 0.20, respectively, whereas **3** and **4** had Rf values of 0.33 and 0.15, respectively. The molecular formulas of **1** and **2** were established as $C_{28}H_{20}N_4O_3$ and $C_{32}H_{26}N_4O_4$ by combination of HR-FABMS and ^{13}C NMR spectral data, respectively. The UV absorption maxima near 252 and 365 nm together with the characteristic low-field chemical shifts of the aromatic protons suggested that these compounds were members of the phenazine class of antibiotics.[21] The IR spectra of **1** and **2** revealed the characteristic absorption band of an ester group (1730 ~ 1735 cm^{-1}) in their structures. The optical rotation values of **1** and **2** were zero. It suggests that they exist in nature as mixtures of enantiomers as has been suggested by FLOSS *et al.*[22] The ^1H NMR spectra of **1** and **2** were shown in Fig. 4.

Biological Activities

Fig. 5 shows the preventive effects of **1** ~ **4** on glutamate toxicity in neuronal N18-RE-105 cells. **1** ~ **4** protected the cells from glutamate toxicity in a dose dependant fashion with EC_{50} values of 0.34, 0.33, 0.15 and 1.28 μM,

Scheme 1. Isolation procedure of phenazostatins A (**1**), B (**2**) and related co-metabolites.

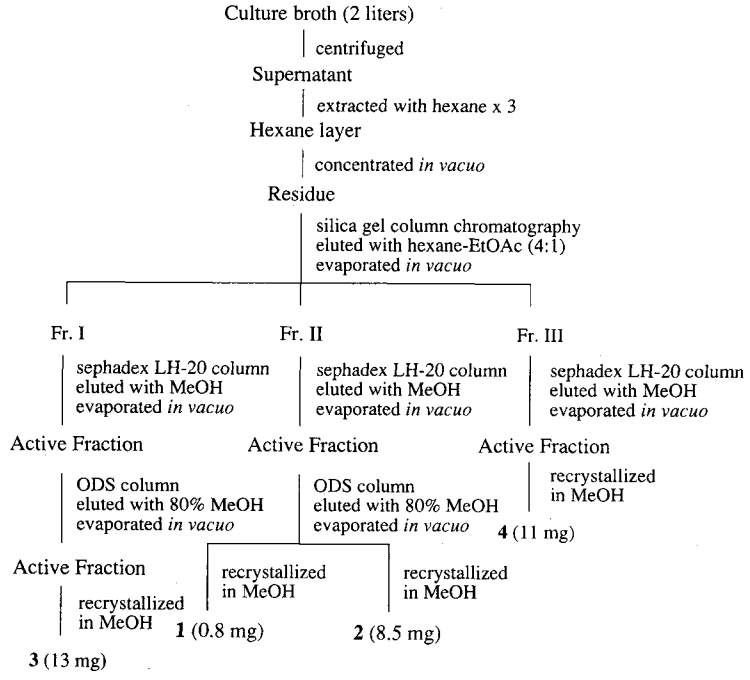


Table 3. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Yellow crystal	Yellow crystal
$[\alpha]_D^{18}$	0° (*c*=0.035, $CHCl_3$)	0° (*c*=0.18, $CHCl_3$)
FAB-MS	461 (M^++1, 29.6%), 279 (100), 265 (78.6), 239 (51.6), 197 (61.9)	531 (M^++1, 98.4%), 266 (80.0), 206 (100)
HRFAB-MS (*m/z*)		
found	461.1626 $(M+H)^+$	531.2040 $(M+H)^+$
calcd.	461.1614	531.2034
Molecular formula	$C_{28}H_{20}N_4O_3$	$C_{32}H_{26}N_4O_4$
UV λ_{max} nm (ε)(MeOH)	252 (86,000)	253 (89,000)
	365 (18,000)	366 (19,000)
IR(KBr)γ cm^{-1}	1730, 1440, 1270, 1190, 1030, 760	1735, 1530, 1280, 1265, 1240, 1190, 1040, 750
Solubility		
soluble	$CHCl_3$, EtOAc, Me_2CO, DMSO	$CHCl_3$, EtOAc, Me_2CO, DMSO
slightly soluble	MeOH, CH_3CN	MeOH, CH_3CN
insoluble	H_2O, *n*-Hexane	H_2O, *n*-Hexane
TLC (Rf)[a]	0.23	0.20
HPLC (R_t)[b] (minute)	9.7	8.5

[a] Silica gel TLC (Kieselgel $60F_{254}$, Merck); solvent, n-Hexane - EtOAc (3:1).

[b] Column, Cosmosil C_{18} (4.6 x 150 mm); solvent, MeOH-H_2O (85:15); flow rate, 1.2 ml/min; detection, UV absorbance at 365 nm.

Fig. 4. ^1H NMR spectra of phenazostatins A (**1**) and B (**2**) in $CDCl_3$ (300 MHz).

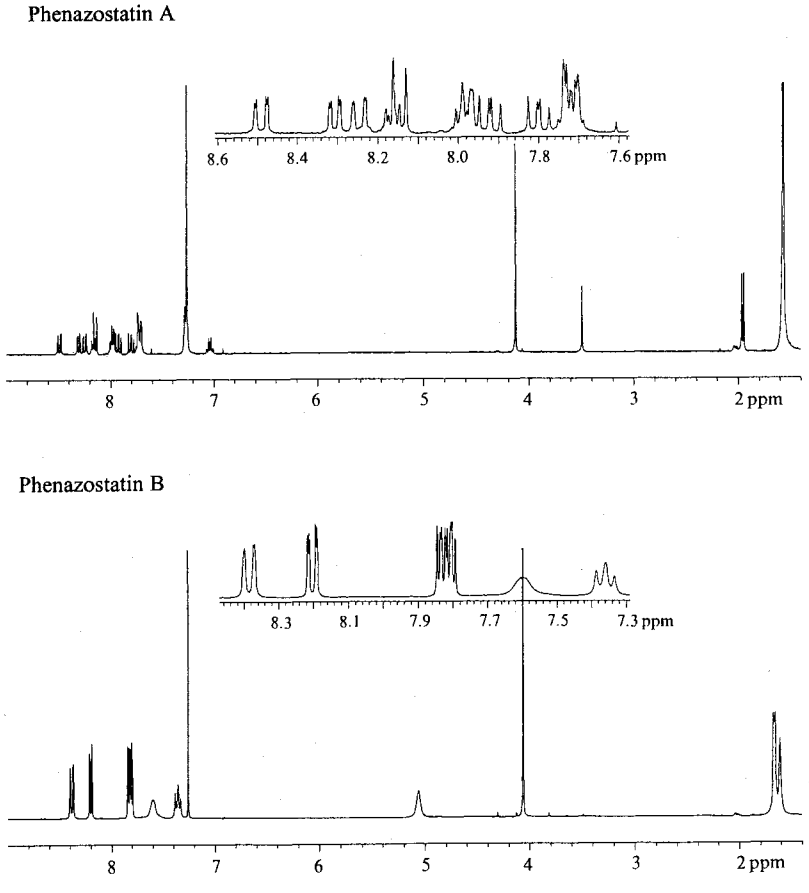


Fig. 5. Effects of **1**~**4** on glutamate toxicity in N18-RE-105 cells and cell viability.

1 (□), **2** (●), **3** (△), **4** (■), idebenone (○).

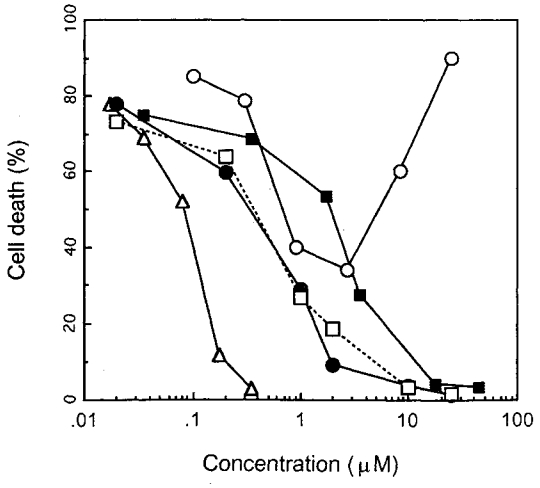


Fig. 6. Inhibitory activity of **1**~**4** against lipid peroxidation in rat liver microsomes.

1 (□), **2** (●), **3** (△), **4** (■), vitamin E (○).

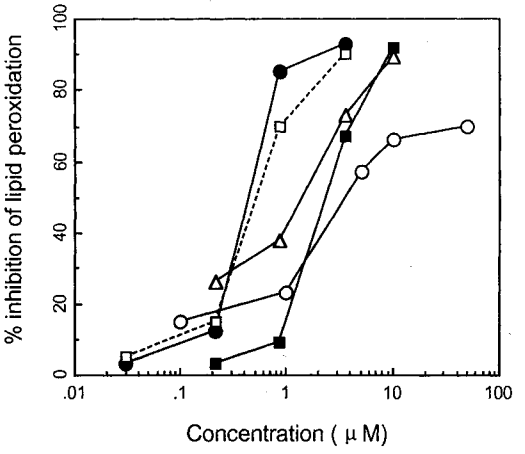


Table 4. Preventive effect against glutamate toxicity (EC_{50}), cytotoxicity (IC_{50}), and free radical scavenging activity (EC_{50}) of **1**~**4**.

Compounds	N18-RE-105		Rat liver microsomes
	EC_{50}(μM)	IC_{50}(μM)	EC_{50}(μM)
1	0.34	>100	0.51
2	0.33	>100	0.44
3	0.15	>100	1.42
4	1.28	>100	2.60
Vitamin E	3.73	>100	4.21
Idebenone	0.72	4.9	4.10

respectively. The inhibition activity of **1** and **2** was around 2-times higher than that of idebenone[23], being used as a brain protective agent. The EC_{50} and IC_{50} values of **1**~**4** in the cell assay are summerized in Table 4. **1**~**4** did not show cytotoxicity at 100 μM while idebenone exhibited strong cytotoxicity with an IC_{50} value of 4.9 μM in this assay system.

For the purpose of evaluating the antioxidative activity of **1**~**4**, the inhibitory activity of these compounds against lipid peroxidation in rat liver microsome was investigated. As shown in Fig. 6, **1**~**4** also inhibited lipid peroxidation induced by free radicals in rat liver microsomes. The inhibition was dose-dependant. **1** and **2** each showed about the same activity with IC_{50} values of 0.51 and 0.44 μM, respectively; which showed about 10-times higher than that of vitamin E. **1** and **2** showed stronger activity than **3** (1.42) and **4** (2.60 μM) with simpler phenazine ring system.

Discussion

In the course of screening for glutamate toxicity inhibitors, we discovered the novel metabolites, phenazostatins A and B, which were isolated from the culture broth of *Streptomyces* sp. 833. Phenazostatins A and B contain an unique diphenazine skeleton which is only found in the antibiotic esmeraldins A and B.[26] Phenazostatin B was previously reported by UMEZAWA *et al.*[27] as an inhibitor of phosphodiesterase, but its structural elucidation was undescribed. FLOSS *et al.*[22] had demonstrated that the esmeraldins, the diphenazine compounds, were biosynthesized from shikimic acid through the intermediate precursor, 6-aceto-phenazine-1-carboxylic acid (*i.e.* a demethylated compound of **3**). Thus, **3** could be the biosynthetic precursor of phenazostatins A (**1**) and B (**2**). Some phenazine compounds of microbial origin such as aestivophoenins[7], benthocyanins[28], phenazoviridin[29], and benthophoenin[30] have been reported as free radical scavengers. It has been known that the glutamate toxicity in N18-RE-105 cells is mainly caused by inhibition of cystine uptake, followed by glutathione depletion and consequently oxidative stress.[24,25] Since phenazostatins A and B exhibited correlated activities against lipid peroxidation and glutamate toxicity, it is very likely that the inhibition of glutamate toxicity is mediated through their antioxidative activity.

Acknowledgments

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