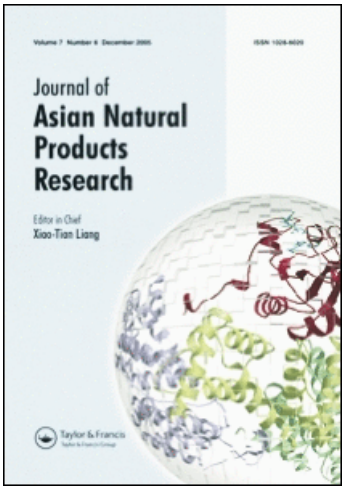

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

A Novel Neuronal Cell Protecting Substance Mescengricin Produced by *Streptomyces griseoflavus*

Kazuo Shin-ya[a]; Jung-Sik Kim[a]; Kazuo Furihata[b]; Yoichi Hayakawa[a]; Haruo Seto
[a] Institute of Molecular and Cellular Biosciences, [b] Department of Applied Biological Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

To cite this Article Shin-ya, Kazuo , Kim, Jung-Sik , Furihata, Kazuo , Hayakawa, Yoichi and Seto, Haruo(2000) 'A Novel Neuronal Cell Protecting Substance Mescengricin Produced by *Streptomyces griseoflavus*', Journal of Asian Natural Products Research, 2: 2, 121 — 132
To link to this Article: DOI: 10.1080/10286020008039901
URL: http://dx.doi.org/10.1080/10286020008039901

JANPR, Vol. 2, pp. 121–132
Reprints available directly from the publisher
Photocopying permitted by license only

A NOVEL NEURONAL CELL PROTECTING SUBSTANCE MESCENGRICIN PRODUCED BY *STREPTOMYCES GRISEOFLAVUS*

KAZUO SHIN-YA[a,†], JUNG-SIK KIM[a,†], KAZUO FURIHATA[b], YOICHI HAYAKAWA[a] and HARUO SETO[a,*]

[a]*Institute of Molecular and Cellular Biosciences*, [b]*Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*

(Received 22 June 1999; Revised 16 August 1999; In final form 20 August 1999)

Neuronal cell death in brain ischemia reperfusion injury such as stroke was induced by L-glutamate toxicity (Choi, D.W. *J. Neurosci.* 1990, **10**, 2493–2501; Coyle, J.T. and Puttfarcken, P. *Science*, 1993, **262**, 689–695). In the course of our screening for neuronal cell protecting substances of microbial origin, we isolated a novel compound designated mescengricin from *Streptomyces griseoflavus* 2853-SVS4 (Kim, J.-S., Shin-ya, K., Furihata, K., Hayakawa, Y. and Seto, H. *Tetrahedron Lett.* 1997, **38**, 3431–3434). The structure of mescengricin was determined by a variety of NMR experiments such as HMBC, D-HMBC (Furihata, K., Seto, H. *Tetrahedron Lett.* 1995, **36**, 2817–2820), ^{1}H–^{15}N HMBC (^{15}N-HMBC). It possesses an α-carboline structure substituted by a glycerol-ester and a hydroxydihydropyrone. Mescengricin protected chick primary mesencephalic neurons from L-glutamate toxicity with EC_{50} value 6.0 nM.

Keywords: Brain ischemia; Glutamate toxicity; Mesencephalon; Neuroprotection

INTRODUCTION

Brain ischemia injury such as stroke victims ranks among the top killers in industrialized nations. By attacking the brain, stroke hits at what makes us human, killing or disabling an apparently healthy person in minutes. In the

* Corresponding author. Tel.: +81-3-5841-7839. Fax: +81-3-5841-8485.
E-mail: haseto@imcbns.iam.u-tokyo.ac.jp.
† The first two authors contributed equally to this work.

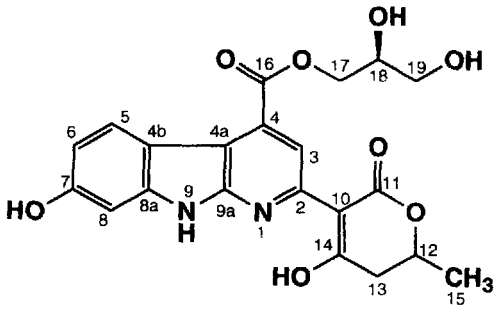

FIGURE 1 Structure of mescengricin (1).

USA, according to the National Institutes of Health (NIH), the condition is the third leading cause of death and the most common cause of adult disability. Of approximately 500,000 new victims each year, roughly 30 percent die, and 20–30 percent become severely and permanently disabled. Others suffer from paralysis, impaired cognition, and speech disabilities with reduced coordination, visual disturbance, loss of sensation or some combination of these effects. Physical therapy can help many people make the best of their remaining capabilities, but cannot repair the brain damage itself. Thus, the need for medical intervention is great, both because of the seriousness of the disorder and its prevalence.

It is well accepted that a major neurotransmitter in the central nervous system is L-glutamate, which is extensively released during brain ischemia and plays an important role in subsequent neuronal cell death through intracellular biochemical cascades [1,2]. Therefore, inhibitors of glutamate-toxicity are expected to be effective against brain ischemia reperfusion injury. In the course of our screening for inhibitors of L-glutamate toxicity using chick primary mesencephalic neuronal cells as an *in vitro* ischemia model, we isolated a novel neuronal protecting substance mescengricin (1) from *Streptomyces griseoflavus* 2853-SVS4 (Fig. 1) [3].

RESULTS AND DISCUSSION

Taxonomy and Fermentation

The substrate mycelium of strain 2853-SVS4 did not fragment. The aerial mycelium irregularly branched on the long main stem and terminated

spirales (3–5 rotation), forming spore chains with 10–20 or more spores per chain. The spores were ellipsoidal (0.4–0.5 × 1.0–1.2 μm) and their surface was spiny. The culture showed no special morphology such as sporangia, whirls or sclerotia. Whole cell hydrolyzate of strain 2853-SVS4 contained LL-diaminopimelic acid. Accordingly, the cell wall of the strain was classified as type I.

The cultural and physiological properties of strain 2853-SVS4 are as follows. Mature aerial mycelia corresponded to the green color series. The reverse side of the colony was light yellow to light reddish orange. No soluble pigment was observed. The results of these morphological and chemotaxonomic studies indicate that strain 2942-SVS3 belongs to the genus *Streptomyces*. Among the species of *Streptomyces* described in BERGEY's Manual [5], strain 2853-SVS4 appeared to be most closely related to *Streptomyces griseoflavus*. Thus, the strain 2853-SVS4 was identified as *Streptomyces griseoflavus* 2853-SVS4.

The mescengricin producing microorganism was inoculated into seed tubes containing 15 ml of the seed medium consisting of soluble starch 1.0%, molasses 1.0%, meat extract 1.0% and polypepton 1.0% (pH 7.0 before sterilization) and incubated on a reciprocal shaker at 27°C for 2 days. The seed culture at 2% was transferred into 500-ml Erlenmeyer flasks containing 100 ml of the producing medium consisting of glycerol 2.0%, molasses 0.5%, casein 0.5% and polypepton 0.1% and calcium carbonate 0.4% (pH 7.2 before sterilization). The flasks were incubated on a rotary shaker at 27°C for 5 days. This culture (600 ml) was further transferred into a 50-l jar fermenter containing 30 l of the same producing medium, and the cultivation was carried out at 27°C for 3 days with agitation 350 rpm and aeration at 30 l per minute.

Isolation

The mycelial cake collected by centrifugation from the whole fermentation broth (90 l) was stirred with 10 l of acetone. The solvent extract was concentrated *in vacuo* to a small volume and active materials were extracted 3 times with each 1.5 l of ethyl acetate. The organic fraction was dried over Na_2SO_4 and concentrated to dryness. The oily residue was dissolved in $CHCl_3$ and 6 times volume of *n*-hexane was added. The precipitates were collected and applied to a silica gel column (50∅ × 400 mm), which was developed with $CHCl_3$–MeOH (7 : 1). The pooled active eluate was concentrated to dryness. The active eluate was then applied to a Toyopearl HW-40F

column and eluted with 100% MeOH. A pure sample of **1** (10 mg) was finally obtained as a reddish brown powder by HPLC using a PEGASIL ODS column (Senshu-Pak, 20⌀ × 250 mm) developed with 60% MeOH.

Structure Elucidation

The physico-chemical properties of **1** are summarized in Table I. The molecular formula of **1** was established as $C_{21}H_{20}N_2O_8$ by HRFAB-MS. IR absorptions at 1730 and 1300 cm^{-1} implied the presence of an ester function.

The ^1H- and ^{13}C-NMR spectral data of **1** are summarized in Table II. One dimensional ^1H- and ^{13}C-NMR spectral data and the phase-sensitive

TABLE I Physico-chemical properties of mescengricin

Appearance	Reddish brown powder
MP	247 ~ 249°C (dec.)
$[\alpha]_D^{20}$	33° (*c* 0.04, MeOH)
Molecular formula	$C_{21}H_{20}N_2O_8$
HRFAB-MS (*m/z*)	
Found	429.1324 (M + H)$^+$
Calcd	429.1298
UV λ_{max}^{MeOH} nm (ε)	210 (10,500), 256 (5,200)
	284 (6,400), 400 (4,500)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	238 (10,000), 410 (4,800)
IR ν_{max} (KBr) cm^{-1}	3400, 1730, 1630, 1300

TABLE II ^1H- and ^{13}C-NMR spectral data for mescengricin

No.	δ_C	δ_H	*No.*	δ_C	δ_H
2	149.1		12	71.0	4.56 (m)
3	112.8	8.57 (br.s)	13	38.4	2.58 (dd, J = 18, 2 Hz),
4	131.6				2.79 (dd, J = 18, 12 Hz)
4a	111.7		14	181.8	
4b	111.9		15	20.2	1.37 (d, J 6 Hz)
5	126.0	8.42 (dd, J = 9, 2 Hz)	16	167.6	
6	110.1	6.74 (dd, J = 9, 2 Hz)	17	68.0	4.37 (ddd, J = 11, 11, 4 Hz),
7	157.9				4.51 (ddd, J = 11, 9, 6 Hz)
8	96.8	6.89 (d, J = 2 Hz)	18	70.6	3.87 (m)
8a	141.4		19	63.9	3.48 (m)
9a	147.4		18-OH		5.08 (br.s)
10	95.8		19-OH		4.76 (br.s)
11	165.9				

^1H-NMR and ^{13}C-NMR spectra were taken in DMSO-d_6 at 500 and 125 MHz, respectively.

DQF spectral data revealed the presence of a 1,2,4-trisubstituted benzene substructure and two spin systems from methyl protons 15-H (1.37 ppm) to methylene protons 13-H (2.58, 2.79 ppm) through a methine proton 12-H (4.56 ppm), and from oxygenated methylene protons 17-H (4.37, 4.51 ppm) to hydroxyl methylene protons 19-H (3.48 ppm) through a hydroxymethine proton 18-H (3.87 ppm) as shown in Figs. 2 and 3.

Since an aromatic proton 3-H (8.57 ppm) playing a key role in the structure elucidation was observed as a very broad signal, only a few $^{1}H-^{13}C$ correlations were observed in the HMBC spectrum of **1**. This problem was overcome by preparation of a free acid derivative (**2**) lacking the glycerol-ester side chain, whose molecular formula was determined by HRFAB-MS as $C_{18}H_{14}N_2O_6$ ($(M+H)^+$, found: 353.0800 (+2.6 mmu)). In the ^{1}H-NMR spectrum of **2**, the aromatic proton 3-H (8.67 ppm) was observed as a sharp signal together with three exchangeable proton signals (9.90, 12.40 and 18.10 ppm). Therefore, the structure determination was carried out by analyzing the NMR spectral data of **2** (Table III).

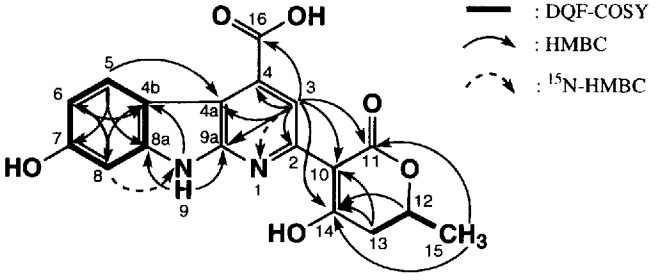

FIGURE 2 NMR spectral analyses of **2**.

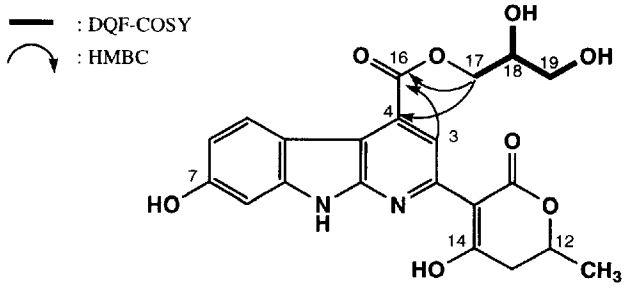

FIGURE 3 NMR spectral analyses of **1**.

TABLE III ^{1}H- and ^{13}C-NMR spectral data for **2**

No.	δ_C	δ_H	*No.*	δ_C	δ_H
2	148.4		10	95.4	
3	112.4	8.67 (s)	11	165.7	
4	134.0		12	70.0	4.58 (m)
4a	112.2		13	38.1	2.63 (dd, $J=17, 3$ Hz),
4b	112.0				2.79 (dd, $J=17, 12$ Hz)
5	126.2	8.45 (d, $J=9$ Hz)	14	181.8	
6	110.3	6.75 (dd, $J=9, 2$ Hz)	15	20.2	1.37 (d, $J=6$ Hz)
7	157.9		16	167.6	
8	96.8	6.89 (d, $J=2$ Hz)	7-OH		9.90 (br.s)
8a	141.0		9-NH		12.40 (br.s)
9a	146.3		14-OH		18.10 (br.s)

^{1}H-NMR and ^{13}C-NMR spectra were taken in DMSO-d_6 at 500 and 125 MHz, respectively.

In the HMBC spectrum of **2**, long-range couplings were detected from the aromatic proton 3-H to quaternary carbons C-2 (112.4 ppm), C-4 (134.0 ppm) and C-4a (112.2 ppm). A long-range coupling from 3-H to a carbonyl carbon C-16 (167.6 ppm) revealed the linkage of C-16 to C-4. Additional long-range couplings were observed from methylene protons 13-H (2.63, 2.79 ppm) to a quaternary carbon C-10 (95.4 ppm) and to an oxygenated sp^2 carbon C-14 (181.8 ppm) which was in turn long-range coupled to the methine proton 12-H (4.58 ppm) and the methyl proton 15-H (1.37 ppm). This methyl proton (15-H) was also long-range coupled to an ester carbonyl carbon C-11 (165.7 ppm). The ^{13}C chemical shifts of C-10, C-11 and C-14, and the correlations cited above established a γ-lactone moiety as shown in Fig. 2.

Furthermore, long-range couplings from the aromatic proton 3-H to the quaternary carbons C-10, C-11 and C-14 in the decoupled-HMBC (D-HMBC) spectrum [4] revealed the position of the γ-lactone moiety on the α-carboline structure.

In addition to these $^{1}H-^{13}C$ correlations, an ^{15}N-HMBC experiment proved a long-range coupling between 3-H and a pyridinium nitrogen N-1 (207 ppm). These results established a substituted pyridine moiety as shown in Fig. 2.

The connectivity between the trisubstituted benzene ring and the substituted pyridine moiety was determined as follows. In the D-HMBC spectrum of **2**, an exchangeable proton 9-NH (12.40 ppm) was long-range coupled to quaternary carbons C-4b (112.0 ppm), C-8a (141.0 ppm) and C-9a (146.3 ppm), the last one being long-range coupled through four-bonds to the aromatic proton 3-H.

Furthermore, a long-range coupling between an aromatic proton 5-H (8.45 ppm) and C-4a was observed. In addition to these correlations, an aromatic proton 8-H (6.89 ppm) was long-range coupled to an amine nitrogen N-9 (122 ppm) in the ^{15}N-HMBC spectrum. The remaining substituent on C-7 was determined to be an oxygen according to the ^{13}C chemical shift of C-7 (157.9 ppm). Thus, the structure consisting of an α-carboline chromophore was determined as shown in Fig. 2.

The connectivity between the α-carboline chromophore and the glycerol moiety was revealed by long-range couplings from 3-H to a carbonyl carbon C-16 (167.1 ppm) and from 17-H (4.51 ppm) to C-16 and C-4 (131.6 ppm) in the HMBC spectrum of **1** as shown in Fig. 3. NOEs observed with the methylene protons 17-H to 3-H and 5-H also supported the structure of **1**.

The stereochemistry of the C-18 position of **1** was determined as follows. An acetonide derivative of **1** was prepared by treatment of **1** with 2,2-dimethoxypropane and pyridinium *p*-toluenesulfonate in CH_2Cl_2. The reaction mixture was hydrolyzed with 1 N NaOH at room temperature for 1 h to give a glycerol dimethylketal. The stereochemistry at C-18 position of **1** was determined to be *S* by comparing the optical rotation value of the obtained glycerol dimethylketal $[\alpha]_D^{21} = +9.8°$ ($c = 0.06$, benzene) with that of D-(+)-glycerol dimethylketal (+10.8°, $c = 15.19$) [6]. The stereochemistry of C-12 remains to be established.

Biological Activities

Chick mesencephalic neurons are susceptible to L-glutamate toxicity, which is known to be observed in rat cortical and hippocampal neurons, and are induced cell death by the addition of 100 μM of L-glutamate as shown in Fig. 4(a) (dead neurons are observed in round shape with vanished neurite). Mescengricin (1 μM) completely protected chick mesencephalic neurons from excitotoxicity and its effective dose range was wide with EC_{50} value 6.0 nM (Fig. 4(b) and Fig. 5). The antioxidative agent vitamin E also exhibited the inhibitory activity against L-glutamate toxicity in chick mesencephalic neurons with EC_{50} value 600 nM. Since vitamin E suppressed the L-glutamate toxicity in this system, we investigated the antioxidative activity of **1** as a model to estimate antioxidative activities using N18-RE-105 cells [7]. L-glutamate-induced cell death in N18-RE-105 cells was reported to be caused by oxidative stress [8,9] and effectively suppressed by antioxidants [10–14]. Vitamin E suppressed the L-glutamate toxicity in N18-RE-105 cells with EC_{50} value 57 nM, whereas **1** did not show the activity even at 10 μM.

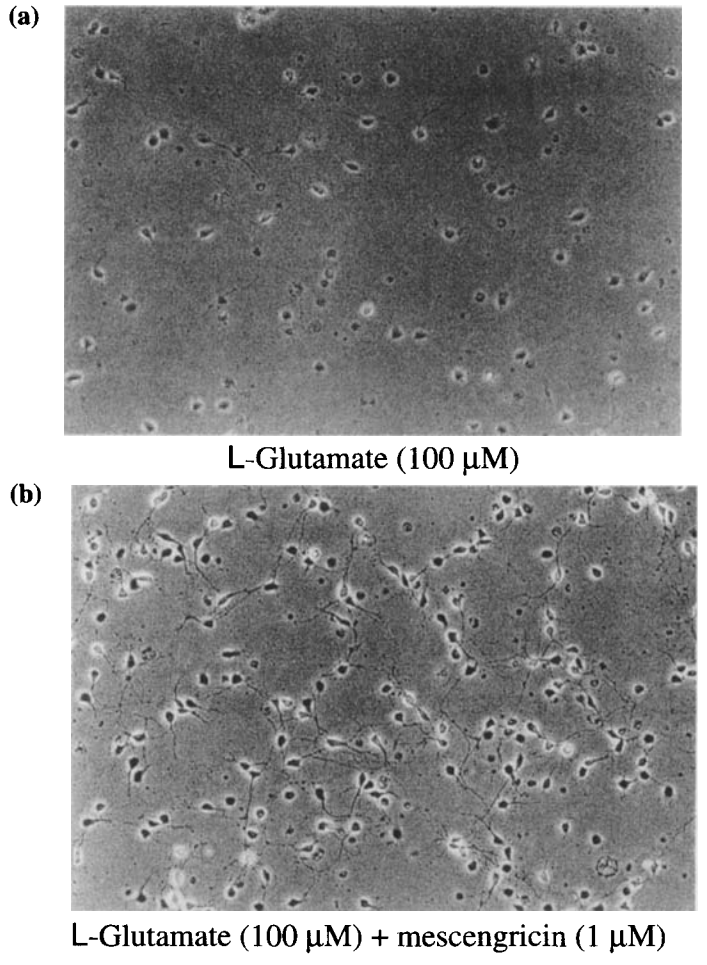

FIGURE 4 Morphological damage produced by exposure to L-glutamate on chick mesencephalic neurons. Phase-contrast photographs follow representative fields of chick mesencephalic neurons. (a) Twenty four hours after L-glutamate (100 μM) treatment showing widespread death with disintegration of neurites. (b) Addition of mescengricin (1 μM) with L-glutamate. Neurons completely survive with prolonged neurite extension.

Since the mechanism of the L-glutamate toxicity in neuronal cells is not fully understood, clarification of the mode of action of **1** is expected to discover a novel mechanism of the L-glutamate toxicity. Further studies on detailed biological activities of **1** are now under way. Since mescengricin is the first natural product with an α-carboline chromophore, its biosynthetic pathway remains to be an interesting target.

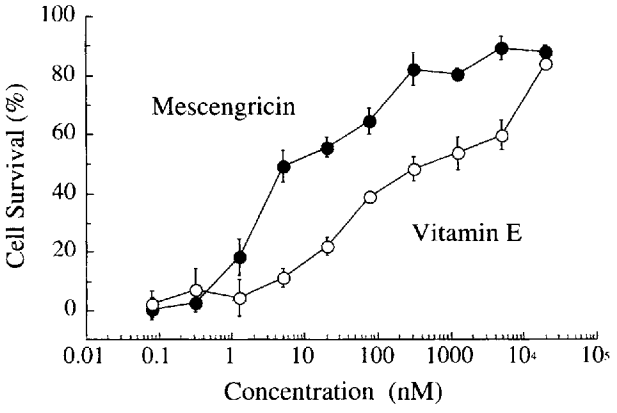

FIGURE 5 Protective effect of **1** against L-glutamate induced excitotoxicity in chick telencephalic neurons.

EXPERIMENTAL SECTION

Spectral Analysis

Mass spectra were recorded on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as a matrix and polyethylene glycol as internal standard. UV and visible spectra were recorded on a Hitachi U-3210 spectrophotometer and IR spectra were recorded on a Jasco A-102 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JNM-A500 spectrometer in DMSO-d_6 solution. Chemical shifts are given in ppm using TMS as internal standard.

Taxonomic Studies

An organism designated 2853-SVS4 was isolated from a soil sample collected in Nagano Prefecture, Japan. Its characterization and identification were carried out mainly according to BERGEY's Manual [5] and the methods described by Shirling and Gottlieb [15]. For the evaluation of cultural characteristics, the strain was incubated for 14 days at 27°C. Cell wall composition was analyzed by the methods of Becker *et al.* [16].

Preparation of a Glycerol Dimethylketal and 2

An acetonide derivative of **1** was prepared by treatment of **1** with 2,2-dimethoxypropane and pyridinium *p*-toluenesulfonate in CH_2Cl_2 for 6 h

at room temperature. The reaction mixture was hydrolyzed with 1 N NaOH at room temperature for 1 h. The glycerol dimethylketal and **2** was purified by silica gel column chromatography using $CHCl_3$-MeOH (30 : 1) as a developing solution.

Cell and Cell Culture

Preparation of Chick Mesencephalic Neurons

Mesencephalic regions from chick embryos, developed to Stage 30 as defined by Hamburger and Hamilton [17], were dissected out in Ca^{2+}-, Mg^{2+}-free Hanks buffer (140 mM NaCl, 5.4 mM KCl, 4 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4 \cdot 12H_2O$, 0.4 mM KH_2PO_4 and 5.5 mM glucose). Tissues from these embryos were treated with 0.25% trypsin and 0.01% DNaseI for 30 min at 37°C. Fetal bovine serum was added to inactivate trypsin, and rinsed with high glucose-DMEM supplemented with GMS-A Supplement (Gibco) and 0.65 μg progesterone. After triturating tissues with gentle pipetting, cells were plated on poly-L-lysine coated 96-well plates containing 100 μl of the same medium at the density of 2.5×10^4 cells/cm^2. After culturing for 3 days, cells were treated with 100 μM L-glutamate to induce cell death. Cytotoxicity was monitored routinely by a phase-contrast microscope (AXIOVART135, ZEISS), and quantified by visible cell counting by using the trypan blue exclusion method.

Preparation of N18-RE-105 Cells

N18-RE-105 hybrid cells (mouse neuroblastoma clone N18TG-2 × Fisher rat 18-day embryonic neural retina) were grown in a Dulbecco's modified Eagle's medium supplemented with 0.1 mM hypoxanthine, 0.04 mM aminopterin, 0.14 mM thymidine and 10% heat-inactivated fetal calf serum. The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. For cytotoxicity studies, cells were plated at 6.25×10^3 cells/cm^2. After culturing for 24 h, the medium replaced with the same medium containing 10 mM L-glutamate. Cytotoxicity was monitored after 24 h by a phase-contrast microscope and quantified by the MTT method as well as by the measurement of the activity of lactate dehydrogenase (LDH) released into the culture medium from degenerated cells.

Cytotoxic Assay

MTT assay, LDH release and visible cell counting using trypan blue exclusion were utilized to quantify the activities of mescengricin and vitamin E.

MTT assay was carried out by treating cells with 5 mg/ml MTT in PBS(−) followed by incubation for 4 h. After removing the medium, equal volume of DMSO was added and absorption values at 570 nm were determined with an automatic microtiter plate reader.

LDH released from degenerated cells into the culture medium was estimated by LDH-Cytotoxic Test (Wako) according to the instruction. Intracellular LDH was recovered by treating viable cells using lysis buffer (0.1 M potassium phosphate buffer (pH 7.0) containing 0.5% Triton X-100). Percentage of the cell death was calculated from the following formula:

$$\% \text{ Cell death} = (\text{LDH activity in the culture medium/total LDH activity}) \times 100.$$

For counting viable cells, cultures in 96-well plates were made up to 0.12% trypan blue, and the viable cells per low magnification field were determined.

Acknowledgments

This work was supported in part by a Grant-in Aid for Scientific Research (C) to K.S. and by Research for the Future, Japan Society for the Promotion of Science to H.S.

References

[1] D.W. Choi, *J. Neurosci.* 1990, **10**, 2493–2501.
[2] J.T. Coyle, P. Puttfarcken, *Science* 1993, **262**, 689–695.
[3] J.-S. Kim, K. Shin-ya, K. Furihata, Y. Hayakawa and H. Seto, *Tetrahedron Lett.* 1997, **38**, 3431–3434.
[4] K. Furihata and H. Seto, *Tetrahedron Lett.* 1995, **36**, 2817–2820.
[5] R. Locci, Actinoplanetes. In: *BERGEY'S Manual of Systematic Bacteriology*, S.T. Williams *et al.* Ed., Williams & Wilkins, 1989, Vol. 4, pp. 2418–2450.
[6] S. Budavari, M.J. O'Neil, P.E. Heckelman and J.F. Kinneary, *The Merck Index* twelfth edition, Merck & Co., Inc. New Jersey, 1996, pp. 889–890.
[7] A.T. Malouf, R.L. Schnaar and J.T. Coyle, *J. Biol. Chem.* 1984, **20**, 12756–12762.
[8] T.H. Murphy, M. Miyamoto, A. Sastre, R.L. Schnaar and J.T. Coyle, *Neuron* 1989, **2**, 1547–1558.
[9] T.H. Murphy, R.L. Schnaar and J.T. Coyle, *FASEB J.* 1990, **4**, 1624–1633.
[10] M. Miyamoto, T.H. Murphy, R.L. Schnaar and J.T. Coyle, *J. Pharmacol. Exp. Ther.* 1989, **250**, 1132–1140.
[11] K. Shin-ya, M. Tanaka, K. Furihata, Y. Hayakawa and H. Seto, *Tetrahedron Lett.* 1993, **34**, 4943–4944.
[12] K. Shin-ya, S. Shmizu, T. Kunigami, K. Furihata, Y. Hayakawa and H.J. Seto, *Antibiotics* 1995, **48**, 574–578.
[13] K. Shin-ya, S. Shimizu, T. Kunigami, K. Furihata, Y. Hayakawa and H.J. Seto, *Antibiotics* 1995, **48**, 1378–1381.

[14] K. Shin-ya, T. Kunigami, J.-S. Kim, K. Furihata, Y. Hayakawa and H. Seto, *Biosci. Biotech. Biochem.* 1997, **61**, 1768–1769.
[15] E.B. Shirling and D. Gottlieb, *Int. J. Syst. Bacteriol.* 1966, **16**, 313–340.
[16] B. Becker, M.P. Lechvalier and H.A. Lechvalier, *Appl. Microbiol.* 1965, **13**, 236–243.
[17] V. Hamburger and H.L. Hamilton, *J. Morphol.* 1951, **88**, 49–92.