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Neuronal Cell Protection Activity of Macrolactin A Produced by *Actinomadura* sp.

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Macrolactin A, 24-membered macrolide, was isolated from the culture broth of *Actinomadura* sp. as a neuronal cell protecting substance. In the cell assay, this compound inhibited glutamate toxicity in N18-RE-105 cells with an EC_{50} value of 0.5 μg/ml.

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 (2, 3). Recent studies indicate that oxygen radicals are produced through a variety of intracellular cascades in such events (3). It was also reported that blockage of glutamate toxicity by free radical scavengers was effective in ameliorating brain ischemia injury (6, 11). Recently, some glutamate toxicity inhibitors of microbial origin such as carquinostatin A (21), lavanduquinocin (22), and aestivophoenins A and B (23) 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 brain ischemia injury, we previously isolated benzastatins A-G (8-10) and phenzazostatins A and B (7, 26). Further investigation have been in the isolation of macrolactin A, 24-membered macrolide, from *Actinomadura* sp. ME-720. Macrolactin A was previously reported by Fenical *et al.* (5) as substance having antibacterial, cytotoxic, and antiviral activities against *Herpes simplex* virus. But, the suppressive activity of macrolactin A against glutamate toxicity in N18-RE-105 cells is reported for the first time in this study. In addition, suppressive activity of the other macrolides including concanamycin, bafilomycin, oligomycin etc. were also investigated. We report herein the taxonomy of the producing strain, isolation, physicochemical properties, structure elucidation and glutamate toxicity suppressive activity of macrolactin A.

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MATERIALS AND METHODS

Microorganisms

Strain ME-720 was isolated from a soil sample collected in Jechun, Chungcheongbook-Do, Korea. The taxonomic studies were carried out as described by the International Streptomyces Project (ISP) (24, 25). 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 using a Guide to Color Standards (Nihon, Shikisai Co., Ltd). Physiological properties including the utilization of carbon sources were examined using the method of Pridham and Gottlieb (18). The type of diaminopimelic acid (DAP) isomers in the cell wall were analyzed according to the methods of Becker *et al.* (1).

Medium and Cultivation

A loopful of strain ME-720 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 macrolactin, 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 at the same conditions.

Inhibitory Activity against Glutamate Toxicity in N 18-RE-105 Cells

N18-RE-105 cells (17) (mouse neuroblastoma clone N 18TG-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 in 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 h, the medium was removed and replaced with a medium containing 10 mM L-glutamate and/or drugs. Cytotoxicity was quantified after treatment for 24 h 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 a 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. EC50 value is the drug concentration necessary to reduce glutamate-induced cell death by 50% (12).

Inhibitory Activity against Lipid Peroxidation in Rat Liver Microsomes

Rat liver microsomes were prepared according to the method of Ohkawa *et al.* (13) 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))×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.

Instrumental Analysis

UV-visible spectra were recorded on a Shimazu UV-260 spectrophotometer in MeOH. Infrared spectra were obtained on a Laser Precision Analect RFX-65 FT-IR spectrometer. Optical rotations were measured on a Polartronic polarimeter. ESI (electrospray ionization)-MS spectra were measured on a Fisons VG Quattro 400 spectrometer. NMR spectra were recorded on Varian UNITY 600 spectrometer. Chemical shifts are given in ppm using TMS as an internal standard. Silica gel (Merck Kieselgel 60, 70~230 mesh) and silica TLC plates (Si gel 60 F_{254}) were purchased from Merck Company.

RESULTS AND DISCUSSION

Taxonomic Studies of the Producing Strain

The strain ME-720 was cultured in various ISP media and the characteristics are summarized in Table 1. The aerial mycelia grew abundantly on yeast extract-malt extract agar and inorganic salts-starch agar, but didn't grow on peptone-yeast extract iron agar and glucose-asparagine agar. The aerial mass color was gray. The substrate mycelia grew abundantly on all ISP media and were pale yellow or yellowish brown in color. Sclerotium, sporangium, and zoospores were not observed. The spore chains were observed to be *retiflexible* type. The spores were cylindrical in shape and had a smooth surface (Fig. 1). The isomer of DAP in the whole cell hydrolysates of strain ME-720 was determined to be the

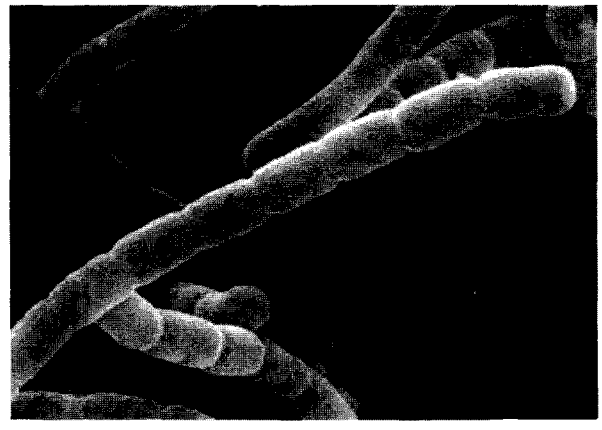

Fig. 1. Scanning electron micrograph of spore chains of strain ME-720 on ISP-2 agar incubated at 27°C for 2 weeks (×20,000).
Bar represents 1 μm.

Table 1. Cultural characteristics of strain ME-720.

Media	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP medium 2)	Good	Abundant, gray	Yellowish brown	None
Oatmeal agar (ISP medium 3)	Good	Abundant, gray	Pale yellow	None
Inorganic salt starch agar (ISP medium 4)	Good	Abundant, greenish gray	Yellowish brown	None
Glycerol-asparagine agar (ISP medium 5)	Moderate	Moderate, gray	Pale yellow	None
Peptone-yeast extract agar (ISP medium 6)	Poor	Poor, N.D.*	Pale yellow	Greenish yellow
Tyrosine agar (ISP medium 7)	Good	Abundant, gray	Pale yellow	None
Glucose-asparagine agar	Poor	Poor, N.D.	Pinkish yellow	None
Nutrient agar	Good	Moderate, Gray	Pale yellow	None
Bennet agar	Moderate	Poor, N.D.	Pale yellow	None

*N.D., Not Detected.

meso-form. The physiological characteristics and the utilization of carbohydrates are shown in Table 2. Melanoid pigments were observed. The cultural characteristics of strain ME-720 were similar to those of *Streptomyces*, but its DAP type in the cell wall was meso. Thus, strain ME-720 was determined to belong to the genus *Actinomadura* (13, 16).

Isolation Procedure of Compound ME-720

The culture supernatant obtained from the culture broth (2 liters) was extracted with an equal volume of ethyl acetate three times and the ethyl acetate layer was concentrated *in vacuo*. The crude extract was subjected to silica gel (Merck art No 7734.9025) column chromatography followed by elution with $CHCl_3$-MeOH (30 : 1). The active fraction was concentrated *in vacuo* and applied to a sephadex LH-20 column, which was developed with methanol. The active eluate was further purified by a reverse phase HPLC column (22.6×300 mm, Phenomenex C_{18}, U.S.A.) chromatography with a photodiode array detector. The column was eluted with MeOH-H_2O (4 : 1) to afford ME-720 (3.2 mg) as a white powder.

Physico-chemical Properties and Structure Elucidation

Physico-chemical properties of ME-720 are summerized in Table 3. ME-720 is soluble in methanol, chloroform, ethyl acetate, and acetone, and insoluble in water and *n*-hexane. ME-720 showed absorption maxima at 229 and 262 nm in the UV spectrum, suggesting the presence of α, β, γ, δ-unsaturated ester group in its structure. The IR spectrum also suggested the presence of an ester group (1697 cm^{-1}) in its structure. The molecular formular of ME-720 was determined to be $C_{24}H_{34}O_5$ by ESI-MS, ^1H and ^{13}C NMR spectral data. The ^1H and ^{13}C NMR spectral data of ME-720 are shown in Table 4. The ^{13}C NMR spectrum exhibited 24 signals composed

Table 2. Taxonomic characteristics of strain ME-720.

DAP type	meso
Spore chain	*Rectiflexibiles*
Spore surface	Smooth
Aerial mass color	Gray
Soluble pigment	Negative
Melanoid pigment	Positive
NaCl tolerence (%)	4%
Starch hydrolysis	Negative
Milk peptonization	Negative
Hydrolysis of skim milk	Positive
Carbon utilization*	
D-Glucose	+
D-Fructose	+
Cellulose	±
Inositol	±
D-Mannitol	±
Raffinose	±
D-Xylose	+
L-Arabinose	+
Cellobiose	+
D-Galactose	+
Inulin	±
Melibiose	+
L-Rhamnose	±
Sucrose	±

*: +, utilized; ±, doubtful.

Table 3. Physico-chemical properties of ME-720.

Appearance	White powder
Molecular formula	$C_{24}H_{34}O_5$
$[\alpha]_D^{18}$ (MeOH)	-20° (*c* 0.1, MeOH)
ESI-MS (*m/z*)	425 $(M+Na)^+$
UV(MeOH) λ_{max} nm (ε)	229 (48500), 262 (18200)
IR($CHCl_3$)γ cm^{-1}	3555-3200, 1697, 1680, 1642
TLC (Rf)	0.32[a]
	0.29[b]
Solubility	
Soluble	MeOH, $CHCl_3$
	Acetone, Ethyl acetate
Insoluble	H_2O, *n*-Hexane

[a] Merck, Kieselgel 60 F_{254} : $CHCl_3$: MeOH=10 : 1. [b] RP-18 F_{254}s: MeOH-H_2O=4 : 1.

Table 4. ^{13}C and ^1H NMR data of ME-720 in $CDCl_3$.

No.	^{13}C (150 MHz)	^1H (600 MHz)
1	166.3 s	–
2	118.0 d	5.59 (d, 11.4)
3	142.7 d	6.54 (dd, 11.4, 11.5)
4	129.8 d	7.20 (dd, 11.5, 14.6)
5	139.4 d	6.08 (dd, 7.8, 14.6)
6	41.5 d	2.48 (m)
7	71.3 d	4.34 (m)
8	136.0 d	5.76 (dd, 5.3, 15.0)
9	124.9 d	6.57 (dd, 10.6, 15.0)
10	130.4 d	6.12 (dd, 10.6, 11.2)
11	127.5 d	5.51 (dd, 9.0, 10.6)
12	35.4 t	2.41 (m)
		2.49 (m)
13	69.6 d	3.99 (m)
14	40.8 t	1.75 (m)
15	70.1 d	4.52 (m)
16	132.8 d	5.60 (m)
17	130.7 d	6.19 (dd, 10.5, 15.3)
18	129.9 d	6.02 (dd, 10.5, 14.2)
19	135.0 d	5.66 (m)
20	32.0 t	2.11 (m)
		2.17 (m)
21	24.4 t	1.49 (m)
22	35.0 t	1.56 (m)
		1.66 (m)
23	70.9 d	5.03 (m)
24	19.9 q	1.27 (m)

The assignments were aided by ^1H-^1H COSY, DEPT, HMQC and HMBC.

of a methyl carbon, 6 methylene carbons, 4 oxygenated methine carbons, 12 olefinic methine carbons, and an ester carbonyl carbon. The structure of ME-720 was determined based on the ^{1}H-^{1}H, ^{13}C-^{1}H connectivity, and ^{1}H-^{13}C long range couplings which were elucidated by ^{1}H-^{1}H COSY, HMQC, and HMBC spectra, respectively, together with DEPT spectra (Fig. 2). The configurations of double bonds at C-2, C-4, C-8, C-10, C-16, and C-18 were assigned to be *Z*, *E*, *E*, *Z*, *E*, and *E*, respectively, on the basis of NOE data and their coupling constants.

The planar structure and all ^{13}C NMR assignments of ME-720 established independently are identical with macrolactin A which was previously isolated from a deep-sea bacterium (5). The absolute stereochemistry of macrolactin A was determined by Fenical *et al.* through chemical studies (19). The good agreements of ^{13}C NMR data of ME-720 with macrolactin A together with similarity of the optical rotation value $[\alpha]_D$=-20°(*c* 0.1, MeOH) of ME-720 with the reported value [-9.6°(*c* 1.86, MeOH)] of macrolactin A suggests that the absolute stereochemistry of ME-720 was the same as that of macrolactin A.

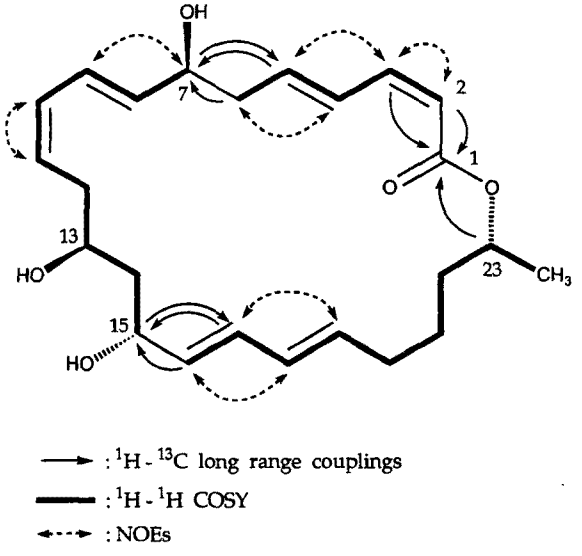

Fig. 2. Structure of ME-720.

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

Fig. 3 shows the preventive effects of macrolactin A against glutamate toxicity in neuronal N18-RE-105 cells. Macrolactin A protected the cells from glutamate toxicity in a dose dependant fashion with an EC_{50} value of 0.5 μg/ml. The inhibition activity of macrolactin A was 3-times higher than that of vitamin E and was half that of idebenone (20), being used as a brain protective agent. Suppressive activity of macrolactin A against glutamate toxicity in N18-RE-105 cells was reported for the first time in this study. Glutamate toxicity suppressive activity of the other macrolides including concanamycin A, anhydroaglycon of concanamycin A, anhydroaglycon of concanamycin B, bafilomycin C1, oligomycin, and avermactin were also investgated (Table 5). Our findings were that they all showed suppressive activity against glutamate toxicity in N18-RE-105 cells. Especially, bafi-

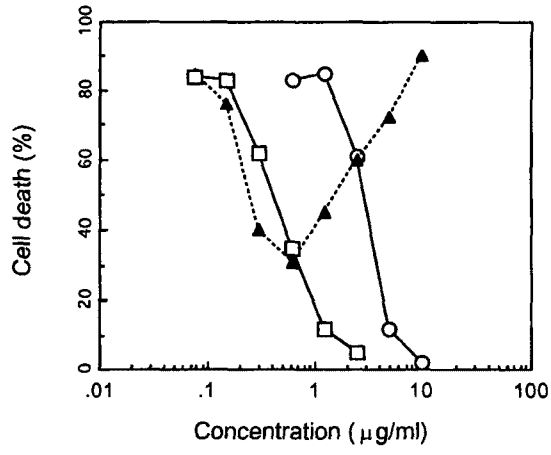

Fig. 3. Effects of macrolactin A on glutamate toxicity in N18-RE-105 cells and cell viability.
Macolactin A (□), Vitamin E (○), Idebenone (▲).

Table 5. Effect of macrolide compounds on glutamate toxicity and their free radical scavenging activity.

Compounds	N18-RE-105 cells			Rat liver microsomes
	EC_{50}[a]	IC_{50}[a]	IC_{50}/EC_{50}[a]	EC_{50}[a]
Macrolactin A	0.50	N.T.[b]	N.T.[b]	> 500
Concanamycin A	0.0025	25	10000	> 500
Anhydroaglycon of concanamycin A	0.0009	25	27000	> 500
Anhydroaglycon of concanamycin B	0.0001	25	250000	> 500
Bafilomycin C_1	0.0001	5	50000	N.T.[b]
Oligomycin	1.62	> 50	> 30.8	> 100
Avermectin	1.55	20	12.9	> 100
Vitamin E	1.50	> 86	57.3	1.68
Idebenone	0.24	1.67	6.96	1.39

[a]μg/ml (ppm). [b]N.T., Not Tested.

lomycin C1 and anhydroaglycon of concanamycin B both exhibited very strong activity with an EC_{50} value of 0.0001 μg/ml which was 2.4×10^3 times higher than idebenone. Anhydroaglycon of concanamycin B showed weak cytotoxicity with an IC_{50} value of 25 μg/ml which was 15 times lower than that of idebenone. It has been shown 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 (14, 15). Since most compounds having suppressive activity against glutamate toxicity in N18-RE-105 cells also were known to have antioxidative activity (4, 7-10, 21-23, 26), the antioxidative activity of macrolactin A and other macrolides were investigated by analyzing their lipid peroxidation inhibitory activity in rat liver microsomes. They showed no lipid peroxidation inhibitory activity as expected from their structures. It is necessary to study the action mechanism of these macrolide further in the future.

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