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NOTE

Indanostatin, a new neuroprotective compound from *Streptomyces* sp.

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Cerebral ischemia is a leading cause of death and long-term disabilities worldwide. In brain ischemia, blood flow disruptions limit the supply of oxygen and glucose to neurons, initiating excitotoxic events. These include activation of glutamate receptors and release of excess glutamate. They induce neuron depolarization and significant increase of intracellular calcium, which activates multiple intracellular death pathways.[1] Accumulation of extracellular glutamate also inhibits cystine-glutamate exchanger, resulting in depletion of the intracellular antioxidant glutathione.[2,3] In such conditions, reactive oxygen species are generated and implicated in neuronal cell death.[4]

C6 glioma cells were originally derived from rat neural tumors induced by *N*-nitrosomethylurea.[5] The cells still maintain the characteristics of glial cells and undergo cell death when exposed to glutamate. Thus, they provide a good model for evaluating neuroprotective activity against glutamate toxicity. Several chemicals including antioxidants have been reported to inhibit glutamate-induced cytotoxicity in C6 cells.[6–8] In this study, microbial metabolites were screened to find neuroprotective compounds against glutamate toxicity. As a result, a new active compound designated indanostatin (**1**) was isolated from the cultured broth of *Streptomyces* sp. RAI20.

The molecular formula of indanostatin (**1**) was determined to be $C_{13}H_{12}O_6$ by high-resolution ESI-MS. ^{13}C and ^{1}H NMR data for **1** are summarized in Table 1. All one-bond ^{1}H–^{13}C connectivities were confirmed by the HMQC[9] spectrum. An HMBC[10] experiment revealed ^{1}H–^{13}C long-range correlations from two phenolic hydroxy protons (4-OH and 7-OH) to a couple of three aromatic carbons (C-3a, C-4, C-5; C-6, C-7, C-7a), from a singlet methyl (H_3-11) to C-4, C-5 and C-6, and from an aromatic proton (H-6) to C-4, C-7 and C-7a, which constructed a 2-methylhydroquinone ring (Figure 1). Long-range couplings from 2-OH to C-1, C-2, C-3 and C-8 connected a hydroxylated quaternary carbon (C-2) to C-1, C-3 and C-8. The existence of a 2-oxopropyl group on C-2 was required by HMBCs from H_2-8 and H_3-10 to C-9. A four-bond correlation between H-6 and C-1 joined the two partial structures to identify the structure of **1** as 4,7-dihydroxy-5-methyl-2-(2-oxopropyl)indan-1,3-dione. The indan skeleton is rarely found in natural products. As 1,3-indanone-containing metabolites, peltatone A[11] and caribbazoins A and B[12] have been isolated from an insectivorous plant (*Drosera peltata* var. *lunata*) and a sponge (*Cliona caribboea*), respectively. To our knowledge, this is the first reported 1,3-indanone from bacteria.

The neuroprotective activity of indanostatin (**1**) was examined by the MTT method using C6 rat glioma cells. When C6 cells were treated with 100 mM glutamate for 24 h, about 80% of the cells underwent cell death. The compound partially protected C6 cells against glutamate toxicity with an EC_{50} of 130 nM as shown in Figure 2. In this condition, α-tocopherol inhibited the glutamate-induced cytotoxicity with an EC_{50} of 47 nM. Indanostatin, however, exhibited no protective activity on N18-RE-105 rat primary retina-mouse neuroblastoma hybrid cells[13,14] at less than 40 μM. Further biological studies are now under way.

EXPERIMENTAL PROCEDURE

General experimental procedure

UV and visible spectra were measured on a Shimadzu UV-1700 spectrometer. Mass and IR spectra were obtained on a Varian 910-MS spectrometer in the ESI mode and a PerkinElmer Spectrum 100 FT-IR spectrometer in the ATR (attenuated total reflection) mode. NMR spectra were obtained on a JEOL JNM-LA400 spectrometer with ^{1}H NMR at 400 MHz and with ^{13}C NMR at 100 MHz. Chemical shifts are given in p.p.m. relative to dimethyl sulfoxide at 2.49 p.p.m. for ^{1}H NMR and 39.7 p.p.m. for ^{13}C NMR.

Producing organism

Strain RAI20 was isolated from a soil sample collected at Nagoya Castle Park, Nagoya, Japan. Genomic DNA was purified by using Genomic-tip and buffer set (Qiagen, Hilden, Germany). The 16S rRNA gene fragment was amplified by PCR using primers with additional *Xba*I and *Hin*dIII sites (5′-GCTCTAGAA-GAGTTTGATCCTGGCTCAG-3′ and 5′-ACCAAGCTTAAGGAGGTGATC-CAGCCGCA-3′).[15] After heating at 98 °C for 1 min, PCR was performed for 30 cycles (98 °C 10 s, 57 °C 30 s, 68 °C 2 min) using KOD Plus ver. 2 DNA

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Table 1 NMR spectroscopic data (400 MHz, DMSO-d_6) for indanostatin (1)

Position	δ_C	δ_H
1	196.5	
2	72.8	
3	200.4	
3a	124.0	
4	146.6	
5	137.0	
6	127.1	7.11 s
7	148.7	
7a	122.1	
8	47.6	3.17 s 2H
9	206.3	
10	29.4	2.04 s 3H
11	15.9	2.22 s 3H
2-OH		6.45 s
4-OH		9.18 s
7-OH		10.19 s

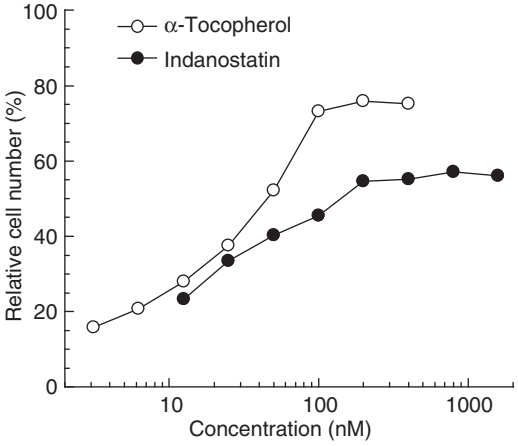

Figure 2 Neuroprotective activities of indanostatin and α-tocopherol against glutamate toxicity. C6 cells were cultured with various concentrations of indanostatin or α-tocopherol in the presence of 100 mM glutamate for 24 h and then the relative cell numbers were measured by the MTT method.

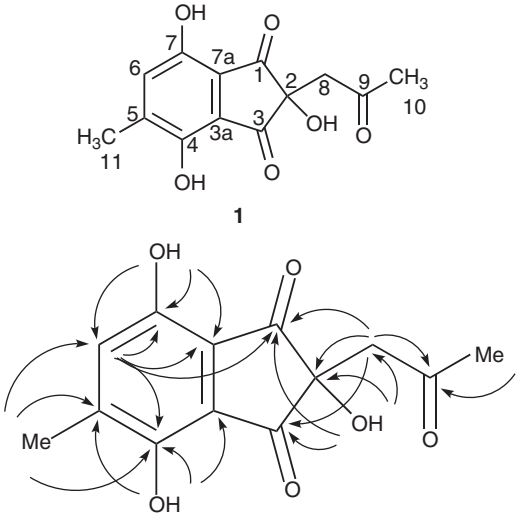

Figure 1 Structure and HMBC analysis of indanostatin (**1**).

polymerase (Toyobo, Osaka, Japan). The PCR product was linked with *Xba*I/*Hin*dIII-digested pGEM-11Z (Promega, Madison, WI, USA) by using T4 DNA ligase. DNA sequencing was carried out with an ABI PRISM Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA, USA). The sequence was compared with the bacterial sequence data stored in DDBJ database by using BLAST algorithm.[16] The sequence revealed high sequence identity with *Streptomyces roseochromogenus* subsp. *albocyclini* NBRC 13 828 (99.0%), *Streptomyces myxogenes* NBRC 13 793 (98.9%) and *Streptomyces miharaensis* NBRC 13 791 (98.8%). Accordingly, strain RAI20 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. RAI20. The 16S rRNA gene sequence of *Streptomyces* sp. RAI20 reported here has been deposited in the GenBank, DDBJ and EMBL databases under accession number AB811079.

Fermentation and isolation

The producing organism was cultivated in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of glucose 2.5%, soybean meal 1.5%, dry yeast 0.2% and calcium carbonate 0.4% (pH 6.2, before autoclave) on a rotary shaker at 27 °C for 4 days. The culture broth (2 l) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was applied to a silica gel column, which was washed with chloroform and then eluted with chloroform–methanol (100: 1). The eluate was subjected to HPLC (Senshu Pak PEGASIL ODS, Senshu Scientific, Tokyo, Japan) with 20% methanol—0.2% trifluoroacetic acid. The active fraction was concentrated to dryness to give a yellow powder of indanostatin (16.0 mg).

Indanostatin (**1**): m.p. 123–129 °C (decomposition); $[\alpha]^{25}_{D}$ −21 (*c* 0.36, MeOH); high-resolution ESI-MS *m/z* 287.05265 ($M+Na^+$, calcd for $C_{13}H_{12}O_6Na$, 287.05261); UV λ_{max} (ε) 241 (26 700), 375 (5800) nm in MeOH, 241 (26 500), 375 (5800) nm in 0.01 M HCl—MeOH, 242 (25 400), 462 (7100) nm in 0.01 M NaOH—MeOH; IR (ATR) ν_{max} 3431, 1681, 1619 cm^{-1}; ^1H and ^{13}C spectroscopic data, see Table 1.

Cells and bioassay

C6 cells (IFO50110) were obtained from the Japanese Cancer Research Resources Bank (JCRB) and N18-RE-105 cells were gifted by Dr K Shin-ya (AIST, Japan). C6 cells were maintained in Dulbecco's modified Eagle's medium and N18-RE-105 cells were maintained in the same medium containing 0.1 mM hypoxanthine, 40 nM aminopterin and 0.14 mM thymidine. These culture media were supplemented with 10% heat-inactivated fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. The cells at 5×10^4 cells ml^{-1} were plated and incubated for 24 h with various concentration of samples and 100 mM (C6) or 10 mM (N18-RE-105) of L-glutamic acid. After the cells were treated with 0.5 mg ml^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C, the relative cell number was measured as absorbance at 570 nm.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)