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BRIEF COMMUNICATION

Pyroxazone, a new neuroprotective compound from *Streptomyces* sp. RAN54

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Abstract

A neuroprotective compound designated pyroxazone (**1**) was isolated from the culture broth of *Streptomyces* sp. RAN54. The molecular formula of **1** was established as $C_{18}H_{14}N_2O_5$ by high-resolution FAB-MS. The structure was determined to be a new 2-amino-3*H*-phenoxazin-3-one derivative by NMR spectroscopic analysis. Pyroxazone (**1**) protected C6 rat glioma cells and N18-RE-105 rat primary retina-mouse neuroblastoma hybrid cells from glutamate-induced toxicity with EC_{50}s of 8.2 μM and 1.7 μM, respectively.

Cerebral ischemic diseases are one of the leading causes of death and long-term adult disability. In brain ischemia, release of excess glutamate induces neuron depolarization and significant increase of intracellular calcium, which activates multiple death pathways [1]. Accumulation of extracellular glutamate also inhibits the cystine-glutamate exchanger, resulting in depletion of the intracellular antioxidant glutathione [2, 3]. Subsequently, reactive oxygen species are generated and implicated in neuronal cell death. C6 rat glioma cells display some neuronal characteristics and undergo cell death when exposed to glutamate [4, 5]. Thus, C6 cells are a good model for evaluating neuroprotective activity against glutamate-induced toxicity. In the course of our screening for neuroprotective compounds of microbial origin using C6 cells, a new active compound designated pyroxazone (**1**) was isolated from the culture broth of an actinomycete strain RAN54. This report describes the isolation, structure elucidation, and biological activity of **1**.

Strain RAN54 was isolated from a soil sample collected at Mibu-machi, Shimotsuga-gun, Tochigi Prefecture, Japan. The 16S rRNA gene fragment was amplified by PCR and sequenced [6]. The sequence displayed high similarity to *Streptomyces tendae* ATCC 19812 (99.3%) and *Streptomyces tritolerans* DAS 165 (99.1%). Accordingly, strain RAN54 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. RAN54. The 16S rRNA gene sequence of *Streptomyces* sp. RAN54 has been deposited in the GenBank, DDBJ, and EMBL databases under accession number LC388337.

The producing organism was cultivated in 500-mL Erlenmeyer flasks containing 100 mL of a medium consisting of 2.5% glucose, 1.5% soybean meal (Nisshin Oillio Group, Tokyo, Japan), 0.2% dry yeast (Asahi Food and Healthcare, Tokyo, Japan) and 0.4% calcium carbonate (pH 6.2 before autoclaving). The fermentation was carried out at 27 °C for 4 days on a rotary shaker. The culture broth (1 L) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate at pH 3. The extract was chromatographed on a silica gel column with chloroform-methanol (100:1). The active fraction was evaporated and the dried material was washed with chloroform and methanol. The residue was dissolved in chloroform-methanol (10:1) and concentrated to dryness to give a pure orange powder of **1** (4.6 mg). The purified sample of **1** was readily soluble in dimethyl sulfoxide (DMSO) or alkaline methanol and slightly soluble in chloroform or methanol.

The physico-chemical properties of pyroxazone (**1**) were as follows. M.P. 288–291 °C (decomposition); FAB-MS

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Table 1 NMR spectroscopic data for pyroxazone (**1**) in DMSO-d_6

Position	δ_C	δ_H (J in Hz)
1	18.4	3.16 t 2H (8.0)
2	29.4	2.62 t 2H (8.0)
3	169.8	
4		9.55s
4a	145.0	
5	177.6	
6	103.0	6.38s
6a	148.9	
7a	142.9	
8	114.2	7.40 dd (8.5, 1.0)
9	131.4	7.53 dd (8.5, 7.5)
10	125.7	7.36 dd (7.5, 1.0)
11	140.8	
11a	131.0	
12a	136.2	
12b	116.0	
13	25.7	3.29 t 2H (7.5)
14	34.8	2.64 t 2H (7.5)
15	173.7	

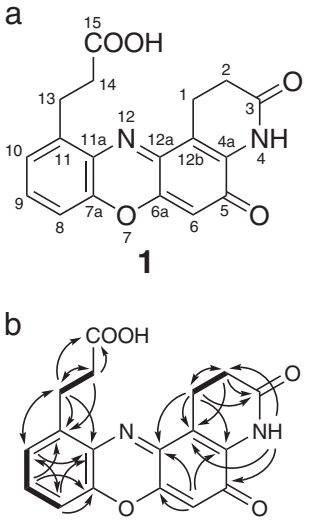

Fig. 1 **a** Structure of pyroxazone (**1**). **b** NMR analyses of pyroxazone (**1**). Bold lines show ^{1}H–^{1}H spin networks and arrows indicate ^{1}H–^{13}C long-range correlations

m/z 339.0984 ($[M+H]^+$, calcd. for $C_{18}H_{15}N_2O_5$, 339.0981); UV λ_{max} (ε) 248 (23,800), 418 (25,700) nm in methanol; IR ν_{max} 3255, 1732, 1659 cm^{-1}.

The molecular formula of **1** was established as $C_{18}H_{14}N_2O_5$ by high-resolution FAB-MS. ^{13}C and ^{1}H NMR data for **1** in DMSO-d_6 are summarized in Table 1. All one-bond ^{1}H–^{13}C connectivities were confirmed by an HMQC experiment. A COSY experiment revealed three proton sequences (H-1-H-2, H-8-H-9-H-10, and H-13-H-14) as shown in Fig. 1b. In the HMBC spectrum, the three *ortho*-coupled aromatic protons (H-8, H-9, and H-10) exhibited long-range correlations to six aromatic carbons (C-7a, C-8, C-9, C-10, C-11, and C-11a) to construct a 1,2,3-trisubstituted benzene ring. Long-range couplings from both H-1 and H-2 to C-3 and C-12b and from both H-13 and H-14 to C-11 and C-15 indicated the presence of two β-carbonylethyl groups located on C-11 and C-12b, respectively. One of the β-carbonylethyl moieties was part of a δ-lactam ring due to long-range correlations from an amide proton (H-4) to C-2 and C-12b. The second aromatic ring with a semi-quinone structure was required by long-range couplings from H-1 to C-4a and C-12a, from H-6 to C-4a, C-6a, and C-12a, and from H-4 to a carbonyl carbon (C-5). The orange color (λ_{max} 418 nm) of **1** suggested conjugation of the two aromatic rings. The two low-field carbons (C-6a, δ 148.9; C-7a, δ 142.9) were joined by an ether linkage to generate a 2-amino-3*H*-phenoxazin-3-one chromophore. Finally, C-15 was attributed to a carboxylic acid group from the molecular formula and the acidity of **1**, and this assignment completed the structure of **1** as shown in Fig. 1a.

A few types of antibiotics contain the 2-amino-3*H*-phenoxazin-3-one chromophore and they include questiomycin A [7, 8], actinomycins [9] and chandrananimycins [10]. Among them, only chandrananimycin C has the same tetracyclic skeleton as **1**, and pyroxazone (**1**) is the first example of 2-amino-3*H*-phenoxazin-3-one derivatives bearing C_3 substituents.

The neuroprotective activity of pyroxazone (**1**) was examined by the MTT method. Cell viability was measured as absorbance at 540 nm, after the cells were treated with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. When C6 rat glioma cells were treated with 100 mM glutamate for 24 h, about 90% of the cells underwent cell death. Pyroxazone (**1**) protected C6 cells from glutamate toxicity with an EC_{50} of 8.2 μM. This compound also showed neuroprotective activity in N18-RE-105 rat primary retina-mouse neuroblastoma hybrid cells [11, 12]. The EC_{50} value of **1** was 1.7 μM in N18-RE-105 cells treated with 10 mM glutamate for 24 h. Further biological studies are now under way.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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