NOTES

A Neuronal Cell Protecting Substance, 4-Demethoxymichigazone, Produced by *Streptomyces halstedii*

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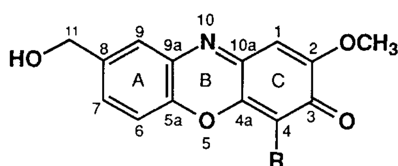
During and after brain ischemia, neuronal degeneration in the hippocampus is mediated largely by an excitatory amino acid, L-glutamic acid. Thus, brain ischemia injury may be expected to be overcome by L-glutamate toxicity suppressors. In the course of our screening program for substances which protect neuronal hybridoma N18-RE-105 cells[1~3] from L-glutamate toxicity, we isolated carquinostatin A[4], lavanduquinocin[5], and aestivophoenins A and B[6]. Further investigation has resulted in the isolation of 4-demethoxymichigazone(8-hydroxymethyl-2-methoxy-3*H*-phenoxazine-3-one) (**1**) from *Streptomyces halstedii* 2832-SVS6. We report herein the isolation, structure, and biological activities of **1**.

Streptomyces halstedii 2832-SVS6, which was isolated from a soil sample collected in Atami, Shizuoka Prefecture, Japan, was cultivated at 27°C in a 60-liter jar fermenter containing 30 liters of a medium consisting of starch 2.5%, soybean meal 1.5%, dry yeast 0.2%, and $CaCO_3$ 0.4%, pH adjusted to 6.2 before sterilization. After fermentation for 72 hours, the culture filtrate was adsorbed on Diaion HP-20. After washing with 50% MeOH, the active fraction was eluted with 100% MeOH and concentrated. The aqueous residue was extracted with EtOAc, and the organic layer was, after drying over Na_2SO_4, evaporated to give an oily residue. This material was subjected to silica gel column chromatography developed with a solvent system of *n*-hexane - EtOAc (1 : 4). The active fraction was then applied to an ODS column (Senshu ODS-SS-1020T) and developed with 60% MeOH. The combined active fraction was purified by preparative HPLC (YMC D-ODS-7) with 20% CH_3CN to give two phenoxazone derivatives, **1** as an orange powder (5.5 mg) and michigazone[7] (**2**) (1.8 mg) as a minor congener (Fig. 1).

Fig. 1. Structures of 4-demethoxymichigazone (**1**) and michigazone (**2**).



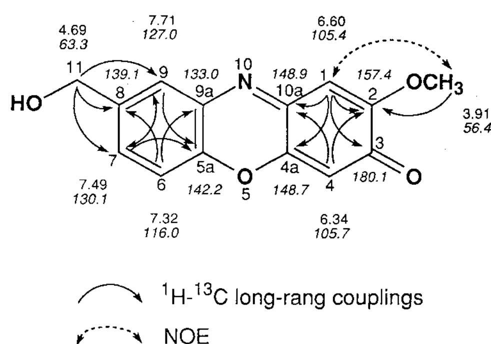
The physico-chemical properties of **1** were as follows: MP 193~194°C; IR ν_{max} (KBr) cm^{-1} 3450, 1620; UV λ_{max} nm (ε) 222 (17,600), 251 (10,600), 390 (11,400), 444 (5,900). The molecular formula of **1** was established by HRFAB-MS as $C_{14}H_{11}NO_4$ (m/z 258.0813 $(M+H)^+$, calcd 258.0767). The UV and visible absorptions of **1** suggested the presence of a phenoxazone nucleus[7,8].

The ^1H and ^{13}C NMR assignments of **1** and **2**, which were made by heteronuclear multiple quantum coherence (HMQC) experiments, are summarized in Table 1. The ^1H and ^{13}C NMR spectral data revealed the presence of a 1,2,4-trisubstituted benzene moiety. The HMBC spectrum showed a hydroxymethyl proton 11-H (4.69 ppm) long-range coupled to C-7 (130.1 ppm), C-8 (139.1 ppm) and C-9 (127.0 ppm). Thus, the hydroxymethyl residue was located at the C-8 position. Assignments of quaternary carbons in the trisubstituted benzene moiety, C-5a (142.2 ppm) and C-9a (133.0 ppm) were established by long-range couplings as shown in Fig. 2. Based on the phenoxazone chromophore structure deduced by UV spectral data, the relationship between two isolated sp^2 protons 1-H (6.60 ppm) and 4-H (6.34 ppm) was assigned to *para* position each other as shown in Fig. 2. One of

Table 1. ^1H and ^{13}C NMR assignments of **1** (500 MHz, in $CDCl_3$).

No.	δ_C	δ_H	
1	105.7	6.60	(s)
2	157.4		
3	180.1		
4	105.7	6.34	(s)
4a	148.7		
5a	142.2		
6	116.0	7.32	(d J = 8.5 Hz)
7	130.1	7.49	(dd J = 2.0 Hz, 8.5 Hz)
8	139.1		
9	127.0	7.71	(d J = 2.0 Hz)
9a	133.0		
10a	148.9		
11	63.3	4.69	(s 2H)
2-OCH_3	56.4	3.91	(s 3H)

Fig. 2. Structure of 4-demethoxymichigazone (1) elucidated by NOE and HMBC experiments.



these isolated protons 1-H was strongly *meta*-coupled to a carbonyl carbon C-3 (180.1 ppm) and C-4a (148.7 ppm), and was additionally *ortho*-coupled to C-2 (157.4 ppm) and C-10a (148.9 ppm). Furthermore, an NOE was observed between 1-H and a methoxy proton (3.91 ppm), which was in turn long-range coupled to C-2. Thus, this methoxy residue was linked to the C-2 position. By considering the presence of the phenoxazone chromophore, C-10a was assumed to be substituted by a nitrogen atom, and the conjugated system consisting of these units was established as shown in Fig. 2. The remaining oxygen atom was linked to C-4a by elimination. Due to a low field shift of 9-H (7.71 ppm) compared with two other aromatic protons 6-H and 7-H (7.32 ppm and 7.49 ppm, respectively) on the same ring system[9,10], 9-H must be at a *peri* position to the nitrogen atom. The ^{13}C chemical shifts of C-5a and C-9a also supported the connectivity between ring A and ring C as shown in Fig. 2. Even though the ^{13}C chemical shifts of **2** were not fully assigned, ^{1}H NMR spectral data of **1** and **2** were quite resembled instead of a methoxy proton (4.05 ppm) in **1**, which is replaced by a singlet aromatic proton (6.34 ppm) in **2**. Thus, the structure of **1** was determined to be 4-demethoxymichigazone. Although the phenoxazone-chromophore containing compounds were reported previously and **1** was synthesized by ACHENBACH *et al.*[8], this paper describes the first isolation of **1** from the nature and the assignments of ^{13}C chemical shifts in phenoxazone moiety.

In the evaluating system we employed[5,11], **1** and **2** protected neuronal hybridoma N18-RE-105 cells from L-glutamate toxicity with ED_{50} values 57.1 and 17.6 nM, respectively. A potent L-glutamate toxicity suppressor carquinostatin A, which was used as a positive control, showed ED_{50} value of 12.7 nM. Since the L-glutamate toxicity in N18-RE-105 cells was reported to be caused mainly by inhibition of cystine uptake[12,13], which is a precursor of the intracellular reducing substance glutathione, we examined the toxicity of a direct inhibitor of glutathione synthesis, buthionine sulfoximine (BSO), on N18-RE-105 cells. The death of N18-RE-105 cells by 500 μM BSO was effectively prevented by **1** and **2** with ED_{50} values 39.8 nM and 18.5 nM, respectively. Further studies on other biological activities are now under way.

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References

1) MALOUF, A. T.; R. L. SCHNAAR & J. T. COYLE: Characterization of glutamic acid neurotransmitter binding site on neuroblastoma hybrid cells. J. Biol. Chem. 20: 12756~12762, 1984
2) MURPHY, T. H.; A. T. MALOUF, R. L. SCHNAAR, A. SASTRE & J. T. COYLE: Calcium dependent glutamate cytotoxicity in a neuronal cell line. Brain Res. 444: 325~332, 1988
3) MURPHY, T. H.; R. L. SCHNAAR, J. T. COYLE & A. SASTRE: Glutamate cytotoxicity in a neuronal cell line is blocked by membrane depolarization. Brain Res. 460: 155~160, 1988
4) SHIN-YA, K.; M. TANAKA, K. FURIHATA, Y. HAYAKAWA & H. SETO: Structure of carquinostain A, a new neuronal cell protecting substance produced by *Streptomyces exfoliatus*. Tetrahedron Lett. 34: 4943~4944, 1993
5) SHIN-YA, K.; S. SHIMIZU, T. KUNIGAMI, K. FURIHATA, K. FURIHATA, Y. HAYAKAWA & H. SETO: A new neuronal cell protecting substance, lavanduquinocin, produced by *Streptomyces viridochromogenes*. J. Antibiotics 48: 574~578, 1995
6) SHIN-YA, K.; S. SHIMIZU, T. KUNIGAMI, K. FURIHATA, Y. HAYAKAWA & H. SETO: Novel neuronal cell protecting substances, aestivophoenins A and B, produced by *Streptomyces purpeofuscus*. J. Antibiotics 48: 1378~1381, 1995
7) WOLF, G.; J. WÖRTH & H. ACHENBACH: Mitomycin and new phenoxazine pigment from *Streptomyces michiganensis*. Arch. Microbiol. 106: 245~249, 1975
8) ACHENBACH, H. & J. WÖRTH: Michigazone, a new phenoxazine pigment from *Streptomyces michiganensis*—structure determination and synthesis. Chem. Ber. 110: 12~22, 1977
9) GERBER, N. N. & H. L. YALE: Structure and syntheses of texazone, 2-(*N*-methylamino)-3*H*-phenoxazin-3-one-8-carboxylic acid, an actinomycete metabolite. J. Antibiotics 36: 688~694, 1983
10) IMAI, S.; A. SHIMAZU, K. FURIHATA, K. FURIHATA, Y. HAYAKAWA & H. SETO: Isolation and structure of a new phenoxazine antibiotic, exfoliazone, produced by *Streptomyces exfoliatus*. J. Antibiotics 43: 1606~1607, 1990
11) MIYAMOTO, M.; T. H. MURPHY, R. L. SCHNAAR & J. T. COYLE: Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. J. Phamacol. Exp. Ther. 250: 1132~1140, 1989
12) TIMOTHY, H. M.; M. MIYAMOTO, A. SASTRE, R. L. SCHNAAR & J. T. COYLE: Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2: 1547~1558, 1989
13) TIMOTHY, H. M.; R. L. SCHNAAR & J. T. COYLE: Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. FASEB J. 4: 1624~1633