NOTES

A Novel Neuronal Cell Protecting Substance, Halxazone, Produced by *Streptomyces halstedii*

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Parkinson's disease (PD) is characterized by selective degeneration of dopaminergic neurons in the substantia nigra. Although the accurate etiology of Parkinson's disease is not clear, one of the hypothetical factors is oxidative stress. The excess administration of L-DOPA, which is an effective drug in PD therapy, results in the worsening of Parkinson's disease, by generating reactive oxygen species (ROS)[1~3]. Thus, the advance of PD may be expected to be overcome by substances which suppress L-DOPA toxicity.

In the course of our screening program for substances which protect rat pheochromocytoma PC12 cells from L-DOPA toxicity, we isolated a novel compound designated as halxazone (8-hydoroxymethyl-1-methoxy-3*H*-phenoxazin-3-one) (**1**) from an actinomycete identified as *Streptomyces halstedii* 4029-SVS1. We report herein the isolation, structure elucidation, and biological activities of **1**.

The producing microorganism, *Streptomyces halstedii* 4029-SVS1, which was isolated from a soil sample collected in Takehara, Hiroshima Prefecture, Japan, was cultivated at 27°C in a 60-liter jar fermenter containing 30 liters of a medium consisting of 2.0% glycerol, 1.0% molasses, 0.5% casein, 0.1% polypepton and 0.4% $CaCO_3$ (pH 7.2) with the agitation rate of 400 rpm and aeration at 30 liters per minute. After fermentation for 72 hours, the culture broth was centrifuged to give a supernatant, which was extracted with an equal volume of EtOAc twice. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a dark brown oil. The oily material was subjected to silica gel column chromatography developed with a solvent system of $CHCl_3$ - MeOH (40 : 1). The active eluate was then applied to Toyopearl HW-40F and developed with MeOH. The active fraction was purified by MPLC using a Senshu Pak PEGASIL ODS column (37 mm i.d.×300 mm) with 40% MeOH. Finally, the pure sample of **1** (3.9 mg) was obtained by HPLC using a Senshu Pak PEGASIL ODS column (20 mm i.d.×250 mm) with 35% MeOH as a congener of michigazone (**2**)[4] and 4-demethoxymichigazone (**3**)[5].

The IR absorption of **1** (mp. 201~202°C) at 3395 and 1640 cm^{-1} indicated the presence of a hydroxyl and a ketone function, respectively. The UV and visible absorptions of **1** (λ_{max} nm (ε) in MeOH: 204 (18,300) 254 (13,500) 365 (10,000) 456 (4,700)) suggested that **1** possesses a phenoxazone nucleus[4~6]. The molecular formula of **1** was established as $C_{14}H_{11}NO_4$ by HRFAB-MS (*m/z* 258.0793 $(M+H)^+$, calcd 258.0766).

The ^1H and ^{13}C NMR data of **1** exhibited 14 carbon and 11 proton signals. These ^1H and ^{13}C assignments are revealed by a heteronuclear multiple-quantum coherence (HMQC) and summarized in Table 1. The HMBC spectra of **1** together with proton spin couplings between 6-H (δ_H 7.29), 7-H (δ_H 7.57) and 9-H (δ_H 7.84) revealed the presence of a 1,2,4-trisubstituted benzene ring. A singlet hydroxymethyl proton signal 11-H (δ_H 4.67) showed ^1H-^{13}C long-range couplings to C-7 (δ_C 132.7), C-8 (δ_C 140.0) and C-9 (δ_C 128.9) in the HMBC spectra. Thus, the hydroxymethyl residue was located at the C-8 position. Other two quaternary carbons in the trisubstituted benzene

Fig. 1. Structure of halxazone (**1**), michigazone (**2**) and 4-demethoxymichigazone (**3**).

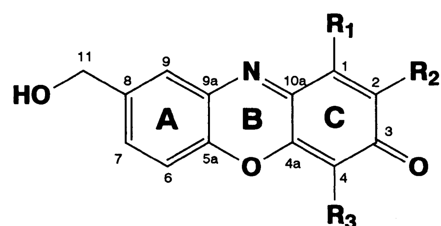

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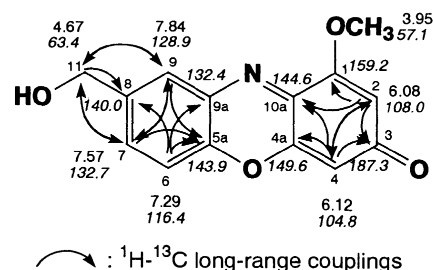
Table 1. ^{13}C and ^{1}H NMR assignments of **1**.

No.	δ_C	δ_H (multiplicity, J = Hz)
1	159.2	
2	108.0	6.08 (d J = 2.0 Hz)
3	187.3	
4	104.8	6.12 (d J = 2.0 Hz)
4a	149.6	
5a	143.9	
6	116.4	7.29 (d J = 8.5Hz)
7	132.7	7.57 (dd J = 2.0, 8.5 Hz)
8	140.0	
9	128.9	7.84 (d J = 2.0Hz)
9a	132.4	
10a	144.6	
11	63.4	4.67 (s 2H)
1-OCH_3	57.1	3.95 (s 3H)

^{1}H and ^{13}C NMR spectra were measured at 500 MHz and 125 MHz, respectively, in $CDCl_3$ + CD_3OD (5 : 1)

Fig. 2. Analyses of ^{13}C-^{1}H long-range couplings in HMBC spectrum of **1**.



ring, C-5a (δ_C 143.9) and C-9a (δ_C 132.4) were assigned by ^{1}H-^{13}C long-range couplings as shown in Fig. 2. Based on the phenoxazone chromophore deduced by UV spectral data *vide supra*, the relationship between two isolated sp^2 protons 2-H (δ_H 6.08) and 4-H (δ_H 6.12) were assigned to the *meta* position from the result of the coupling constant (J_{H-H}=2.0 Hz) as shown in Fig. 2. In the HMBC spectra of **1**, 2-H and 4-H were strongly *meta*-coupled to a quaternary carbon C-10a (δ_C 144.6), and were *ortho*-coupled to a carbonyl carbon C-3 (δ_C 187.3). In addition, 2-H also long-range coupled to a quaternary carbon C-1 (δ_C 159.2), which in turn long-range coupled to a methoxy proton (δ_H 3.95) revealing that the methoxy residue was substituted at C-1. An *ortho*-coupling from 4-H to C-4a (δ_C 149.6) established the assignments of all ^{13}C and ^{1}H signals.

Taking into consideration the presence of the phenoxazone chromophore and molecular formula of **1**, C-10a was deduced to be substituted by a nitrogen atom, and the remaining oxygen atom was linked to C-4a. Thus, the conjugated system consisting of these units was established as shown in Fig. 2. Two possibilities were considered about the connectivity between ring A and ring B. Due to a low field shift of 9-H (δ_H 7.84) compared with two other aromatic protons 6-H (δ_H 7.29) and 7-H (δ_H 7.57) in the same ring system, 9-H must be at a *peri* position to the nitrogen atom[7,8]. The ^{13}C chemical shifts of C-5a and C-9a also supported the connectivity between ring A and ring B as shown in Fig. 2.

The protective effect of **1**, **2** and **3** on L-DOPA toxicity in PC12 cells was examined employing the method of MIGHELI *et al.*[1] after minor modification. **1**, **2** and **3** potentially protected PC12 cells from L-DOPA (50 μM) toxicity with EC_{50} values of 15.4, 10.7 and 30.0 nM, respectively. Since **3** was reported as the neuroprotecting substance which suppressed L-glutamate toxicity in N18-RE-105 cells[9,10], the protective effect of **1** against L-glutamate toxicity was observed. **1** also protected N18-RE-105 cells from L-glutamate toxicity with EC_{50} value of 19.1 nM, which was almost identical to that of **2**. L-Glutamate toxicity in N18-RE-105 cells was considered to be induced by the accumulation of ROS[11~13]. Thus, **1** was deduced to protect PC12 cells from L-DOPA toxicity by exhibiting anti-ROS activity. Detailed studies on biological activities are now under way.

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