Phenazostatin C, a New Diphenazine with Neuronal Cell Protecting Activity from *Streptomyces* sp.

WON-GON KIM, IN-JA RYOO, BONG-SIK YUN, KAZUO SHIN-YA†, HARUO SETO† and ICK-DONG YOO*

Korea Research Institute of Bioscience and Biotechnology, P. O. Box 115, Yusong, Taejon 305-600, Korea
†Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

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L-Glutamate, a major neurotransmitter in the central nervous system, has been known to be extensively released during brain ischemia and induces subsequent neuronal cell death.[1,2] Recent studies indicate that oxygen radicals are produced through a variety of intracellular cascades in such events.[2] It was also reported that blockage of glutamate toxicity by free radical scavengers was effective to ameliorate brain ischemia injury.[3,4] Recently, some glutamate toxicity inhibitors of microbial origin such as carquinostatin A,[5] lavanduquinocin,[6] and aestivophoenins A and B[7] 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 the brain ischemia injury, we previously isolated benzastatins A~G[8~10] and phenazostatins A (**2**) and B.[11,12] Further investigation on metabolites of *Streptomyces* sp. 833 which is the producer of phenazostatins A and B has resulted in isolation of an unique diphenazine compound, phenazostatin C (**1**) (Fig. 1). We report here the isolation, physico-chemical properties, structure determination, and biological activities of **1**.

The culture supernatant obtained from the culture broth (2 liters) was extracted with an equal volume of hexane three times and the hexane layer was concentrated *in vacuo*. The crude extract was subjected to a silica gel (Merck art No 7734.9025) column followed by elution with *n*-hexane - EtOAc (4 : 1). After elution of the fraction containing **2**, another active fraction was successively eluted. The new 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 C-18 (YMC-gel ODS-A Lot No. 51252) column chromatography. The column was eluted with 80% aqueous MeOH to give the active fraction. The active fraction was finally recrystallized in MeOH to afford **1** (2.4 mg) as yellow crystals.

The physico-chemical properties of **1** are summarized in Table 1. **1** is soluble in chloroform, ethyl acetate, acetone, and dimethylsulfoxide, slightly soluble in methanol and acetonitrile, and insoluble in water and *n*-hexane. After TLC on silica gel 60 F_{254} (Merck) with *n*-hexane - EtOAc (3 : 1), **1** showed an Rf value of 0.30 whereas **2** had an Rf value of 0.21. The UV absorption maxima at 249 and 365 nm together with the characteristic low-field chemical shifts of the aromatic protons suggested that this compound was a member of the phenazine class of antibiotics. The IR spectra of **1** revealed the characteristic absorption band of an ester group (1734 cm^{-1}). The optical rotation value of **1** was zero. It suggests that like **2**, **1** exists in nature as a mixture of enantiomers as has been suggested by FLOSS *et al.*[13]

The molecular formula of **1** was determined to be $C_{30}H_{22}N_4O_4$ on the basis of high resolution FAB-MS [$(M+H)^+$, *m/z* 503.1748 (−0.3 mmu error)] in combination

Fig. 1. Structures of phenazostatins C (**1**) and A (**2**).

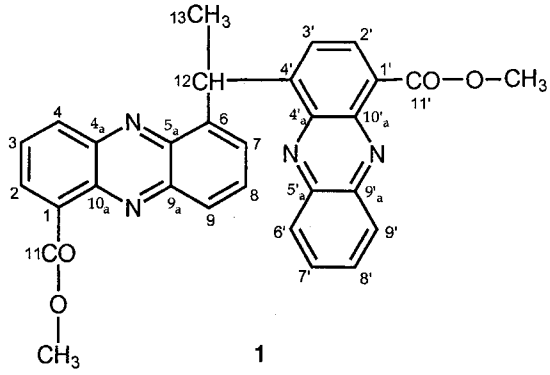


Table 1. Physico-chemical properties of **1**.

Appearance	Yellow crystal
$[\alpha]_D^{18}$	0° (*c* 0.2, $CHCl_3$)
FAB-MS	503 $(M+H)^+$
HRFAB-MS (*m/z*)	
found	503.1748 $(M+H)^+$
calcd.	503.1751
Molecular formula	$C_{30}H_{22}N_4O_4$
UV λ_{max} nm (ε)(MeOH)	249 (109,000)
	365 (26,000)
IR(KBr) ν cm^{-1}	1734, 1435, 1284, 1194,
	1029, 754
Solubility	
soluble	$CHCl_3$, EtOAc, Me_2CO, DMSO
slightly soluble	MeOH, CH_3CN
insoluble	H_2O, *n*-Hexane

with ^1H and ^{13}C NMR data. Together with UV and IR spectral data, the ^1H and ^{13}C NMR spectra of **1** were similar to those of **2** (Table 2), suggesting that **1** also was a diphenazine compound. The difference between **1** and **2** in ^1H and ^{13}C NMR data with HMQC data was that the ^{13}C chemical shift of the methine signal (C-12) of the branched ethyl group was upfield-shifted from 72.2 to 34.0, suggesting that the methine was not oxygenated, and two carboxylic carbons (δ 167.2 and 167.3) and two methoxy groups (δ_H 4.01, 3H, s and 4.10, 3H, s; δ_c 52.6, q and 52.7, q) were appeared instead of the signals corresponding of the methoxycarbonyl group of **2**, suggesting the presence of two methoxycarbonyl groups in **1**. In addition, one more sp^2 quaternary carbon was observed in **1** instead of disappearance of one aromatic methine carbon of **2**, suggesting that the new methoxycarbonyl group could be attached to the phenazine ring. The difference of aromatic proton signals between **1** and **2** in ^1H NMR data with ^1H-^1H COSY was that *ortho*-coupled aromatic protons (δ 7.58, d, J=7.3

Table 2. ^1H and ^{13}C NMR spectral data for **1** and **2**.

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		131.0 s		130.9 s
2	8.18 (1H, m)[a]	132.0 d	8.31	132.2 d
3	7.68 (1H, dd, 8.1, 6.9)	127.8 d	7.92	128.8 d
4	8.02 (1H, dd, 8.1, 0.9)	134.0 d	8.49	133.9 d
4_a		142.0 s		141.9 s
5_a		142.2 s		140.7 s
6		145.5 s		140.6 s
7	7.71 (1H, m)	128.3 d	7.98	126.7 d
8	7.77 (1H, dd, 8.5, 7.0)	130.7 d	7.80	130.5 d
9	8.17 (1H, m)	128.6 d	8.25	129.8 d
9_a		144.0 s		143.6 s
10_a		140.5 s		140.8 s
11		167.2 s		167.2 s
12	7.04 (1H, q, 7.1)	34.0 d	7.04	72.2 d
13	2.00 (3H, d, 7.1)	21.0 q	1.96	23.2 q
11-OCH_3	4.10 (3H, s)	52.7 q	4.13	52.7 q
1'		129.2 s	7.28	107.1 d
2'	8.11 (1H, d, 7.3)	132.2 d		159.2 s
3'	7.58 (1H, d, 7.3)	126.0 d	7.72	126.9 d
4'		151.0 s	8.15	130.4 d
$4'_a$		141.2 s		140.6 s
$5'_a$		142.2 s		143.1 s
6'[b]	7.97 (1H, d, 7.8)	130.0 d	7.99	129.5 d
7'[c]	7.75 (1H, dd, 7.8, 7.0)	130.5 d	7.72	130.4 d
8'[c]	7.82 (1H, dd, 8.4, 7.0)	130.8 d	7.72	129.1 d
9'[b]	8.29 (1H, d, 8.4)	130.2 d	8.16	129.7 d
$9'_a$		143.3 s		141.8 s
$10'_a$		141.1 s		144.9 s
11'		167.3 s		
11'-OCH_3	4.01 (3H, s)	52.6 q		

The numbering was done for comparison.

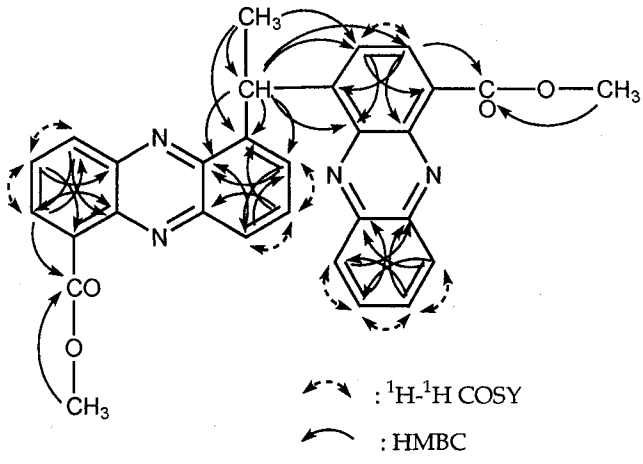
All spectra were recorded at 600 MHz for ^1H and 150 MHz for ^{13}C in $CDCl_3$.

[a] Proton resonance multiplicity and coupling constant (J = Hz) are in parenthesis.

[b, c] Assignments interchangeable.

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

Fig. 2. HMBC and ^{1}H-^{1}H COSY data of **1**.



Hz and 8.11, d, J=7.3 Hz) were observed instead of the AMX aromatic proton spin system of **2**. From these spectral data, it was suggested that the branched ethyl group and the new methoxycarbonyl group could be attached to the phenazine ring in either *ortho*- or *para*-position. The position of these groups was determined by HMBC experiments. Long-range couplings were observed from the methine proton (H-12) to four sp^2 quaternary carbons at δ 142.2 (C-5$_a$), δ 145.5 (C-6), δ 151.0 (C-4′), and δ 141.2 (C-4′$_a$) and two aromatic methine carbons at δ 128.3 (C-7) and δ 126.0 (C-3′). In the ^{1}H-^{1}H COSY, the aromatic proton (δ 7.58, d, J=7.3 Hz) of H-3′ was *ortho*-coupled to H-2′ (δ 8.11, d, J=7.3 Hz) which was, in turn, long-range coupled to the carbonyl carbon at δ 167.3 (C-11′) of the methoxycarbonyl group. In addition, the methyl protons (H-13) of the branched ethyl group (δ 2.0, 3H, d, J=7.1 Hz) were long-range coupled to C-6, C-12, and C-4′. These HMBC indicated that the methine (C-12) and the methoxycarbonyl group (C-11′) should be attached to the benzene moiety of the phenazine ring in the *para*-position as shown in Fig. 1. The remaining structure of **1** was also confirmed by the HMBC and ^{1}H-^{1}H COSY spectral data in Fig. 2.

1 protected neuronal N18-RE-105 cells[14,15] from glutamate toxicity in a dose-dependant fashion with an EC_{50} value of 0.37 μM, which showed about the same activity with that of **2**. The inhibitory activity of **1** was around 2-times higher than that of idebenone (0.7 μM),[16] being used as a brain protective agent. **1** did not show cytotoxicity at 100 μM while idebenone exhibited strong cytotoxicity with an IC_{50} value of 4.9 μM in this assay system. For the purpose of evaluating the antioxidative activity of **1**, the inhibitory activity of **1** against lipid peroxidation[17] in rat liver microsome was investigated. **1** also inhibited lipid peroxidation induced by free radicals in rat liver microsomes in a dose-dependant manner. The IC_{50} value of **1** was 0.6 μM which showed about 6-times higher than that of vitamin E (3.72 μM), a well known antioxidant.

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