COMMUNICATIONS TO THE EDITOR

Novel Neuronal Cell Protecting Substances, Aestivophoenins A and B, Produced by *Streptomyces purpeofuscus*

Sir:

Brain ischemia and subsequent reperfusion injury in stroke victims ranks among the top killers in the industrialized nations. According to the Ministry of Health and Welfare, the condition is the third leading cause of death and the most common cause of adult disability in Japan. Physical therapy can help many peoples to make the best of their remaining capabilities, but cannot repair the brain damage itself. Thus, the need for medical intervention is great, both because of the seriousness of the disorder and its prevalence.

It has been well accepted that the excitatory amino acid, L-glutamate acting as a neurotransmitter in the major part of brain, induces neuronal cell death after brain-ischemic attack[1]. L-Glutamate proved to generate oxygen radicals through a variety of intracellular cascades in such events[2]. Under some conditions, brain-ischemia injury is prevented by free radical scavengers[3]. In the course of our screening for inhibitors of glutamate-toxicity using the neuronal hybridoma N18-RE-105 cells as an *in vitro* ischemia model, we isolated carquinostatin A[4], 4-demethoxymichigazone[5] and lavanduquinocin[6]. Further investigation has resulted in the isolation of novel compounds designated aestivophoenins A and B (**1** and **2**, respectively, Fig. 1).

The aestivophoenins producing organism, identified as *Streptomyces purpeofuscus* 2887-SVS2, was cultivated in a 50-liter jar fermenter containing 30 liters of the medium consisted of glycerol 2.0%, molasses 1.0%, casein 0.5%, polypeptone 0.1% and $CaCO_3$ 0.4% at 27°C for 3 days. The mycelial acetone extract was concentrated to a small volume, and the aqueous residue was adjusted to pH 3, and extracted with EtOAc. The solvent layer was dried over Na_2SO_4 and concentrated to give an oily residue. This material was washed with *n*-hexane and the remaining residue was, after concentration to dryness, subjected to a silica gel column packed with *n*-hexane-EtOAc (4:1). After washing with the same solvent system, the active material was eluted with $CHCl_3$-MeOH (20:1). Further purification of the active eluate by an ODS column (ODS-SS-1020T, Senshu Scientific Co., Ltd.) with 90% methanol gave a mixture of **1** and **2**. Finally, pure **1** and **2** were isolated as orange powders by HPLC using PEGASIL ODS (Senshu-Pak, 20 i.d. × 250 mm) developed with 85% and 90% MeOH, respectively.

The physico-chemical properties of **1** and **2** are summarized in Table 1. Both **1** and **2** showed identical UV and visible spectra, which resembled those of benthophoenin[7], a benzoated phenazine derivative. The molecular formulae of **1** and **2** were established by high-resolution FAB-MS as $C_{31}H_{32}N_2O_7$ and $C_{36}H_{40}N_2O_7$, respectively, suggesting that **2** was an isoprenyl adduct of **1**. The ^1H and ^{13}C NMR spectral data are shown in Table 2. The structures of **1** and **2** were elucidated as follows.

The phase-sensitive DQF spectrum of **1** revealed the

Fig. 1. Structures of aestivophoenins A (**1**) and B (**2**).

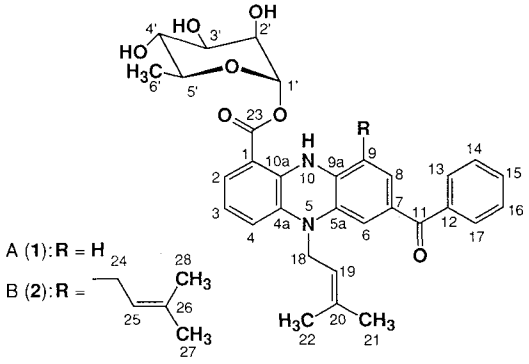


Table 1. Physico-chemical properties of aestivophoenins A (**1**) and B (**2**).

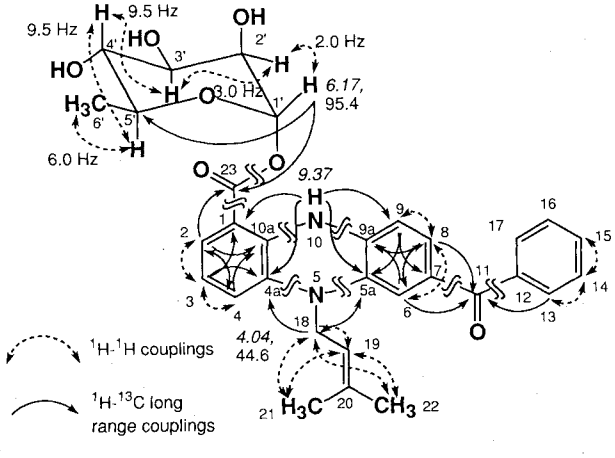
	A	B
Appearance	Orange powder	Orange powder
MP	59 ~ 61°C	63 ~ 64°C
$[\alpha]_D^{20}$	−106.3 (c = 0.005)	−139.8 (c = 0.006)
Molecular formula	$C_{31}H_{32}N_2O_7$	$C_{36}H_{40}N_2O_7$
HRFAB-MS (m/z)		
found	544.2211 $(M)^+$	612.2841 $(M)^+$
calcd	544.2209	612.2835
UV λ_{max}^{MeOH} nm (ε)	229 (28,900), 245 (27,400), 296 (26,000), 367 (4,000), 490 (11,700)	232 (26,900), 245 (25,800), 298 (19,000), 368 (3,300), 495 (9,700)
IR ν_{max} (KBr) cm^{-1}	3450, 3330, 1680, 1260	3450, 3330, 1680, 1260

Table 2. ^{13}C and ^{1}H chemical shifts of aestivophoenins A (**1**) and B (**2**) in acetone-d_6.

No.	A δ_C	A δ_H	B δ_C	B δ_H	No.	A δ_C	A δ_H	B δ_C	B δ_H
1	109.7		109.6		18	44.6	4.04	44.8	3.98
2	122.3	7.08	122.0	7.06	19	119.5	5.08	119.6	5.05
3	121.6	6.55	121.6	6.53	20	137.5		137.4	
4	115.4	6.35	115.0	6.30	21	25.8	1.74	25.7	1.73
4a	136.7		136.7		22	18.0	1.69	18.0	1.65
5a	135.5		135.3		23	166.8		167.1	
6	112.9	6.70	111.4	6.59	24			29.3	3.08
7	132.4		131.5		25			120.6	5.22
8	127.2	7.00	127.4	6.95	26			135.8	
9	112.8	6.50	123.8		27			25.8	1.71
9a	139.1		136.7		28			18.1	1.71
10a	140.5		140.5		1'	95.4	6.17	95.4	6.20
11	194.5		194.6		2'	70.9	3.99	70.9	4.00
12	139.7		139.7		3'	72.3	3.81	72.2	3.82
13,17	129.9	7.68	129.9	7.67	4'	73.2	3.53	73.2	3.53
14,16	129.0	7.51	128.9	7.50	5'	72.1	3.75	72.1	3.77
15	132.2	7.59	132.2	7.59	6'	18.2	1.25	18.2	1.26
					10-NH		9.37		9.50

presence of five substructures; a dimethylallyl residue, 1,2,3- and 1,2,4-trisubstituted benzene rings, a phenyl group and a 6-deoxyhexopyranose moiety as shown in Fig. 2. In the HMBC spectrum of **1**, the terminal methylene proton 18-H (4.04 ppm) of the dimethylallyl residue was long-range coupled to C-4a (136.7 ppm) and C-5a (135.5 ppm) through a nitrogen atom as evidenced by their ^{13}C chemical shifts. Thus, these two trisubstituted benzene rings were connected to each other as shown in Fig. 2. Since an aromatic proton 2-H (7.08 ppm) was long-range coupled to C-23 (166.8 ppm), C-1 (109.7 ppm) was substituted by this carbonyl carbon. Both aromatic protons 6-H (6.70 ppm) and 8-H (7.00 ppm) on the 1,2,4-trisubstituted benzene ring were long-range coupled to a carbonyl carbon C-11 (194.5 ppm), which was, in turn, coupled to aromatic protons 13,17-H (7.68 ppm) on the phenyl group. Since the ^{13}C chemical shifts of C-9a and C-10a were too high to be located at *ortho* positions of nitrogen substituents (139.1 and 140.5 ppm, respectively), these carbons were also assumed to be substituted by a nitrogen atom. Thus, an exchangeable proton 10-H (9.37 ppm) was assigned to an amine which was long-range coupled to C-1, C-4a, C-5a and C-9 (112.8 ppm). The remaining sugar moiety was determined as rhamnose by analyzing the coupling constants from 6'-H (1.25 ppm) to 1'-H (6.17 ppm) through 5'-H (3.75 ppm), 4'-H (3.53 ppm), 3'-H (3.81 ppm) and 2'-H (3.99 ppm). In addition, the anomeric proton 1'-H was coupled to C-5' (72.1 ppm) and an ester carbonyl carbon C-23. From these data, the rhamnose moiety was linked to the ester carbonyl C-23. The other ^{1}H-^{13}C long-range couplings supporting the structure of **1** were as shown in Fig. 2. The stereochemistry at the

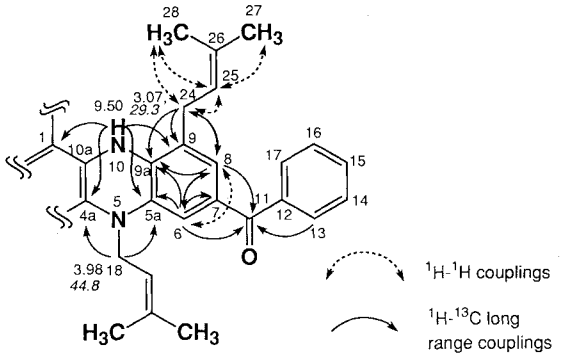
Fig. 2. Structure of **1** elucidated by DQF COSY and HMBC experiments.



anomeric carbon C-1' was elucidated to be α by the ^{13}C-^{1}H coupling constant of C-1' ($^{1}J_{C\text{-}H} = 175.7$ Hz)[8,9]. Thus, the final structure of **1** was determined as shown in Fig. 1. The absolute stereochemistry was established as shown bellow.

The structure of **2** was elucidated by comparing NMR spectral data with those of **1**. As described above, **2** was considered to be a dimethylallyl adduct from its molecular formula. In the ^{1}H NMR spectrum of **2**, one dimethylallyl residue, a 1,2,3-trisubstituted benzene ring, a phenyl group and a rhamnose moiety were preserved, but the signals due to the 1,3,4-trisubstituted benzene ring in **1** was replaced by two *meta*-coupled doublet signals. Furthermore, signals assignable to an additional dimethylallyl residue appeared. In the HMBC spectrum

Fig. 3. ^{1}H-^{1}H and ^{1}H-^{13}C correlations for partial structure of **2** by DQF COSY and HMBC experiments.



of **2**, the terminal methylene proton 24-H (3.08 ppm) was long-range coupled to C-8 (127.4 ppm), C-9 (123.8 ppm) and C-9a (136.7 ppm). In addition, an aromatic proton 8-H (6.95 ppm) which was coupled to a *meta* proton 6-H (6.59 ppm) was long-range coupled to the terminal methylene carbon C-24 (29.3 ppm). These data suggested that this dimethylallyl residue was substituted at the C-9 position as shown in Fig. 3, and the other dimethylallyl residue was located at the N-5 position as in **1**. Other ^{1}H-^{13}C long-range couplings supported the structure of **2**. The stereochemistry of an anomeric carbon C-1′ of **2** was also elucidated to be α based on the ^{13}C-^{1}H coupling constant of C-1′ ($^{1}J_{C\text{-}H}=175.7$ Hz).

In attempt to verify the absolute configuration of the rhamnose moiety, the major component **2** was methanolyzed in 5% HCl-MeOH to give α-methyl rhamnoside which was shown to possess the L configuration by comparison of the optical rotation $[\alpha]_D^{20}=-36.4°$ ($c=0.05$, MeOH) with the reported value ($-62.5°$, $c=2$)[10]. Consequently, the structure of **2** including the absolute stereochemistry was determined as shown in Fig. 1.

In the evaluation system we employed[11,12], **1** and **2** suppressed effectively the toxicity of L-glutamate in N18-RE-105 cells with EC_{50} values 15.0 nM and 6.2 nM, respectively. Furthermore, **1** and **2** also protected rat embryonic primary hippocampal neurons from the L-glutamate toxicity more than 40 nM. The chromophore common to **1** and **2** consists of the benzoated phenazine moiety present in benthocyanins A[13], B, C[14], benthophoenin and phenazoviridin[15]. Since these compounds were reported as potent antioxidants, **1** and **2** are also expected to show an antioxidative activity. Further studies on detailed biological activities and *in vivo* test are now under way.

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