NOTES

A Novel Neuronal Cell Protecting Substance, Aestivophoenin C, Produced by *Streptomyces purpeofuscus*

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The excitatory neurotransmitter, L-glutamate plays a critical role in the neuronal injury that results from cerebral ischemia or head trauma. Following either of these insults the concentration of extracellular L-glutamate is elevated, and then this event induces many processes including an increase in intracellular Ca^{2+} concentration, hyperkalemia, activation of proteases, synthesis of nitric oxide and production of reactive oxygen species[1~3]. These processes finally trigger neuronal cell death. This neuronal degeneration is prevented by glutamate receptor antagonists[4,5] or antioxidants[6,7].

In the course of our screening program for inhibitors of L-glutamate toxicity using neuronal hybridoma N18-RE-105 cells[8] as an *in vitro* brain ischemia model, we isolated carquinostatins A[9], B[10], 4-demethoxymichigazone[11], lavanduquinocin[12] and aestivophoenins A and B[13]. Further investigation has resulted in the isolation of aestivophoenin C (**1**) from *Streptomyces purpeofuscus* 2887-SVS2 as a minor congener of the aestivophoenins. In this paper, we report isolation, structural elucidation and biological activities of **1**.

The aestivophoenins producing organism identified as *Streptomyces purpeofuscus* 2887-SVS2 was cultivated at 27°C in a 50-liter jar fermenter containing 30 liters of a medium consisting of glycerol 2.0%, molasses 1.0%, casein 0.5%, and polypeptone 0.1% and $CaCO_3$ 0.4% (adjusted to 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 separated to supernatant and mycelium by a Sharples centrifugation machine. The mycelial cake was extracted with acetone followed by concentration to a small volume. 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 fraction 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% MeOH as a developing solvent gave a mixture of aestivophoenins. Finally, the pure sample of **1** was isolated by HPLC using PEGASIL ODS developed with 90% MeOH (Senshu-Pak, 20 i.d. × 250 mm) as a yellow powder.

The physico-chemical properties of **1** were as follows: MP 61~62°C; IR ν_{max} (KBr) cm^{-1} 3410, 1690, 1260: UV λ_{max} nm (ε) in MeOH; 247 (24,600), 328 (2,000), 453 (4,100). The molecular formula of **1** was established as

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

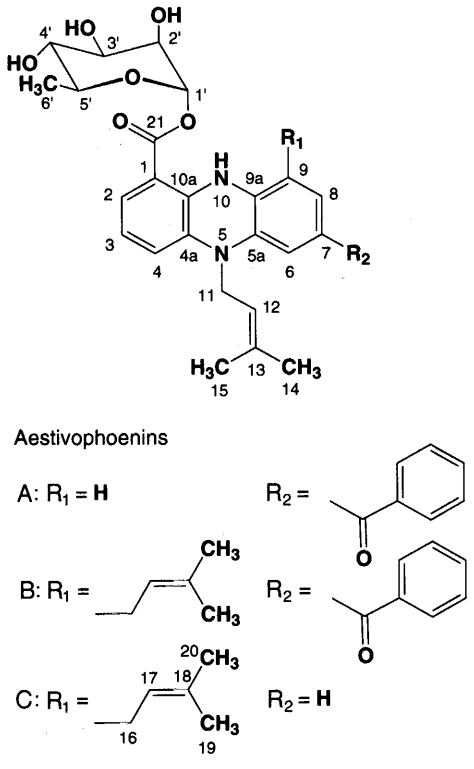


Table 1. ^{13}C and ^{1}H chemical shifts of aestivophoenins B and C (1) in acetone-d_6.

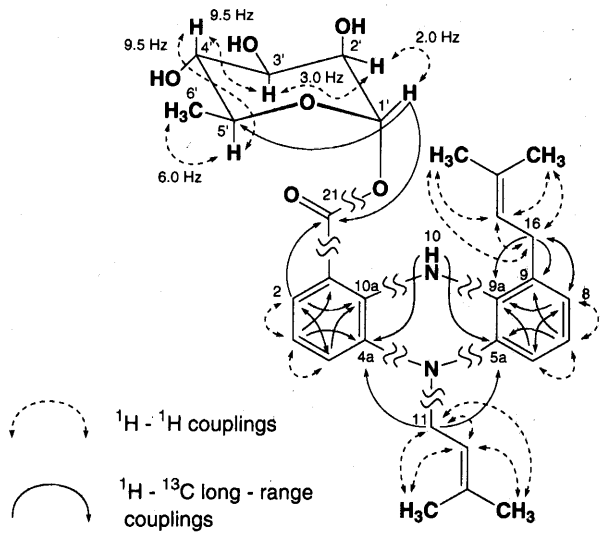
No.	C (1)		B	
	δ_C	δ_H	δ_C	δ_H
1	108.5		109.6	
2	121.7	7.04	122.0	7.06
3	120.3	6.45	121.6	6.53
4	114.5	6.24	115.0	6.30
4a	137.0		136.7	
5a	135.5		135.3	
6	110.6	6.17	111.4	6.59
7	122.9	6.53	131.5	
8	122.9	6.43	127.2	7.00
9	124.9		123.8	
9a	131.5		136.7	
10a	142.6		140.5	
11	44.7	4.09	44.8	3.98
12	120.7	5.07	119.6	5.05
13	136.7		137.4	
14	25.7	1.75	25.7	1.73
15	18.1	1.80	18.0	1.65
16	29.5	3.08	29.3	3.08
17	121.5	5.23	120.6	5.22
18	134.7		135.8	
19	25.8	1.73	25.8	1.71
20	18.1	1.73	18.1	1.71
21	167.1		167.1	
1′	95.1	6.20	95.4	6.20
2′	70.9	3.97	70.9	4.00
3′	72.3	3.81	72.2	3.82
4′	73.2	3.52	73.2	3.53
5′	72.1	3.76	72.1	3.77
6′	18.2	1.24	18.2	1.26
10-NH		9.24		9.50
Benzoly moiety				
1			194.6	
2			139.7	
3, 7			129.9	7.68
4, 6			128.9	7.50
5			132.2	7.59

$C_{29}H_{36}N_2O_6$ by HRFAB-MS (m/z 508.2587 $(M)^+$, calcd 508.2574), which suggested the lack of a benzoyl unit from aestivophoenin B.

The ^{1}H and ^{13}C NMR data of **1** are summarized in Table 1 together with those of aestivophoenin B. The structure of **1** was elucidated by NMR spectral comparison with aestivophoenin B[13]. Analysis of the ^{1}H NMR of **1** revealed two 1,2,3-trisubstituted benzene rings, two dimethylallyl residues and a 6-deoxyhexopyranose moiety. In addition, an exchangeable proton 10-NH (9.24 ppm) assignable to an amine proton was observed. In the HMBC spectrum of **1**, the terminal methylene protons 11-H (4.04 ppm) of a dimethylallyl residue, which was reasonably connected to a nitrogen atom based on the ^{13}C chemical shift (44.7 ppm) of the relevant carbon, were long-range coupled to quaternary aromatic carbons C-4a (137.0 ppm) and C-5a (135.5 ppm), which in turn long-range coupled to the amine proton 10-NH. Thus, two 1,2,3-trisubstituted benzene units and these two nitrogen atoms constructed a phenazine skeleton, which was the common chromophore to aestivophoenins A and B.

The substituted positions of two units, an acylated sugar moiety and a dimethylallyl residue, were elucidated as follows. Long-range couplings from terminal methylene protons 16-H (3.08 ppm) of the other dimethylallyl residue to aromatic carbons C-8 (122.9 ppm), C-9 (124.9 ppm) and C-9a (131.5 ppm) and from an aromatic proton

Fig. 2. Structure elucidation of aestivophoenin C (1) by ^{1}H-^{1}H COSY and HMBC experiments.



8-H (6.43 ppm) to the methylene carbon C-16 (29.5 ppm) established the substituted position of the second dimethylallyl residue at C-9. The remaining sugar moiety was determined as rhamnose by analyzing the coupling constants from 5′-H (3.76 ppm) to 1′-H (6.20 ppm) through 4′-H (3.52 ppm), 3′-H (3.81 ppm) and 2′-H (3.97 ppm). These ^{1}H and ^{13}C chemical shifts of this sugar unit were comparable to those of aestivophoenins

A and B. The absolute stereochemistry of rhamnose was considered to be L as shown in a previous paper. The connectivity between the chromophore substructure and the sugar moiety was confirmed by long-range couplings from an ester carbonyl carbon C-21 (167.1 ppm) to 1'-H and 2-H. Thus, the sugar moiety was linked to the C-1 position through the ester carbonyl carbon C-21 as shown in Fig. 2. The structure of aestivophoenin C including absolute stereochemistry was established to be a debenzoyl derivative of aestivophoenin B as shown in Fig. 1.

In the evaluation system we employed[14], **1** and an antioxidant vitamin E protected N18-RE-105 cells from L-glutamate toxicity with EC_{50} values of 18.3 nM and 56.3 nM, respectively. Since L-glutamate toxicity in N18-RE-105 cells was reported to be caused by suppression of the synthesis of the intracellular reducing agent glutathione[15,16] *via* the inhibition of cystine uptake, we examined the protective effect of **1** against buthionine sulfoximine (BSO), a potent inhibitor of glutathione synthetase, toxicity. **1** and vitamin E also suppressed BSO toxicity in N18-RE-105 cells with EC_{50} values 20.2 nM and 44.7 nM, respectively. Since antioxidants such as vitamin E suppressed L-glutamate toxicity in N18-RE-105 cells and the chromophore of **1** which consists of a phenazine skeleton was similar to those of antioxidants such as benthocyanins A[17], B, C[18], benthophoenin[19] and phenazoviridin[20], the mode of action of **1** was attributed to antioxidative activity. Further studies on the detailed biological activities of **1** are now under way.

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