

NOTE

Flaviogeranin, a new neuroprotective compound from *Streptomyces* sp.

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Cerebral ischemic disorders are one of the main causes of death. In brain ischemia, blood flow disruptions limit the supply of oxygen and glucose to neurons, initiating excitotoxic events. These include activation of glutamate receptors and release of excess glutamate inducing neuron depolarization and significant increase of intracellular calcium, which activates multiple intracellular death pathways.¹ Accumulation of extracellular glutamate also inhibits cystine–glutamate exchanger, resulting in depletion of the intracellular antioxidant glutathione.^{2,3} Under such conditions, reactive oxygen species are generated and implicated in neuronal cell death.⁴ In this study, microbial metabolites were screened to find neuroprotective agents against glutamate toxicity.

C6 rat glioma cells undergo cell death when exposed to 100 mM glutamate for 24 h. Thus, they provide a good model for evaluating neuroprotective activity against glutamate toxicity. Our screening using C6 cells resulted in the isolation of a new active compound designated as flaviogeranin (Figure 1) from *Streptomyces* sp. RAC226.

The producing organism was cultivated in 500 ml Erlenmeyer flasks containing 100 ml of a medium consisting of glucose (2.5%), soybean meal (1.5%), dry yeast (0.2%) and calcium carbonate (0.4%) (pH 6.2, before autoclave) on a rotary shaker at 27 °C for 5 days. The culture broth (2 l) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was adjusted to pH 3 and extracted with ethyl acetate. The extract was applied to preparative silica gel TLC plates, which was developed with hexane–ethyl acetate–triethylamine (150:50:5). The crude material was subjected to HPLC (PEGASIL ODS, Senshu Scientific, Tokyo, Japan) with 87% methanol and 0.2% trifluoroacetic acid. The active fraction was further purified by XBridge C₁₈ HPLC (Waters, Milford, MA, USA) with 87% methanol and 0.2% triethylamine. The peak fraction was concentrated to dryness to give an orange powder of flaviogeranin (2.2 mg).

The physicochemical properties of flaviogeranin are summarized as follows m.p. 160–165 °C; high-resolution FAB-MS *m/z* 371.1858 (MH⁺, calcd for C₂₂H₂₇O₅, 371.1859); UV λ_{max} (ε) 221 nm (252 000),

265 (125 000), 307 (64 200) and 429 (29 300) in methanol, 220 nm (250 000), 265 (125 000), 307 (64 000) and 429 (29 300) in 0.01 M HCl–methanol, 220 nm (sh, 246 000), 265 (109 000), 307 (59 500) and 429 (25 000) in 0.01 M NaOH–methanol; and IR (ATR) ν_{max} 3090, 3060, 1680 and 1630 per cm.

The molecular formula of flaviogeranin was established as C₂₂H₂₆O₅ by high-resolution FAB-MS. The ¹³C- and ¹H-NMR data for flaviogeranin are summarized in Table 1. All one-bond ¹H–¹³C connectivities were confirmed by an HMQC⁵ experiment. The HMBC⁶ spectrum identified a naphthoquinone chromophore as shown in Figure 2. Long-range correlations from a phenolic hydroxyl (5-OH) to C-4a, C-5 and C-6 and from a singlet methyl (9-H₃) to C-5, C-6 and C-7 revealed the sequence of four aromatic carbons (C-4a, C-5, C-6 and C-7). This substructure was extended to a benzene ring owing to HMBCs from 8-H to C-4a, C-6, C-7 and C-8a. No correlation between 8-H and C-5 arranged them in para positions. A carbonyl carbon (C-1) coupled with 8-H was attached to C-8a. Another carbonyl (C-4) was located at C-4a from intramolecular hydrogen bonding of 5-OH (δ 12.69) to form a 1,4-naphthoquinone skeleton. Correlations from 3-H to the quinone ring carbons except for C-8a required a para orientation between 3-H and C-8a. A methoxy group was substituted at C-2 from their ¹H–¹³C long-range coupling. A dimethylallyl group was constructed by ¹H–¹³C long-range correlations from two allylic methyls (8'-H₃ and 10'-H₃) to C-6' and C-7' and a COSY cross peak between 5'-H₂ and 6'-H (Figure 2). Another isoprene unit was identified by HMBCs from both 9'-H₃ and 4'-H₂ to C-2' and C-3' and a vicinal coupling between 1'-H₂ and 2'-H. The two units were joined from correlations from 4'-H₂ to C-5' and from 5'-H₂ to C-4'. An *E*-configuration for C-2' was deduced from the upfield chemical shift of C-9' (δ 16.7). A geranyl group thus obtained was connected to 7-O of the naphthoquinone chromophore from a long-range correlation between 1'-H₂ and C-7. These results established the structure of flaviogeranin as 7-geranyloxy-5-hydroxy-2-methoxy-6-methyl-1,4-naphthoquinone.

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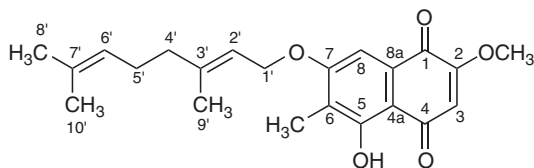


Figure 1 Structure of flaviogeranin.

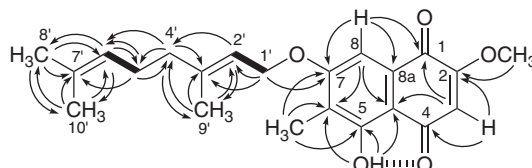


Figure 2 NMR analysis of flaviogeranin. Bold lines indicate ^1H - ^1H couplings and arrows show ^1H - ^{13}C long-range correlations.

Table 1 ^{13}C - and ^1H -NMR data for flaviogeranin in acetone- d_6

No.	δ_{C}	δ_{H} (J=Hz)
1	179.7	
2	162.0	
3	109.8	6.14 s
4	191.3	
4a	109.3	
5	161.3	
6	121.3	
7	162.8	
8	104.1	7.19 s
8a	130.9	
9	8.4	2.11 3H s
1'	66.5	4.82 2H d (6.0)
2'	120.2	5.48 t (6.0)
3'	142.4	
4'	40.2	2.10 2H m
5'	26.9	2.12 2H m
6'	124.6	5.08 t (6.0)
7'	132.1	
8'	25.7	1.61 3H s
9'	16.7	1.82 3H s
10'	17.7	1.57 3H s
2-OCH ₃	57.1	3.93 3H s
5-OH		12.69 s

The neuroprotective activity of flaviogeranin was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The relative cell viability was measured as absorbance at

570 nm, after cells were incubated with 0.5 mg ml⁻¹ of MTT for 4 h. Flaviogeranin prevented cell death in C6 cells treated with 100 mM of glutamate for 24 h with an EC₅₀ of 8.6 nM. This compound also suppressed cell death in N18-RE-105 rat primary retina-mouse neuroblastoma hybrid cells^{7,8} with an EC₅₀ of 360 nM in the presence of 10 mM of glutamate. No cytotoxicity against N18-RE-105 cells was observed with flaviogeranin at concentrations up to 2 μM. Further biological studies are now under way.

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