NOTES

Nerfilin I, a Novel Microbial Metabolite Inducing Neurite Outgrowth of PC12 Cells

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Nerve growth factor (NGF) is a neurotrophic factor essential for survival and functioning of nerve cells[1]. It has emerged as a potential thorough therapeutic for a variety of neurological diseases. However, since the NGF molecule is too large in size to pass through the blood-brain barrier, NGF-like low molecular weight compounds are considered to be good candidates for treating patients with nerve diseases. During the course of our screening for microbial metabolites which induce neurite outgrowth of rat pheochromocytoma cell-line PC12[2], a novel active substance nerfilin I was isolated from the culture broth of *Streptomyces halstedii* 2723-SV2. This paper presents fermentation, purification, physico-chemical properties and structure elucidation of nerfilin I and its congener, nerfilin II.

A preculture of *S. halstedii* 2723-SV2 was inoculated into three 50-liters jar fermentors containing 27 liters of the culture medium consisting of glycerol 2%, molasses 1%, casein 0.5%, polypepton 0.1% and $CaCO_3$ 0.4% (pH 7.0 before sterilization), and fermentation was carried out at 27°C for 24 hours with agitation at 150 rpm and an air flow at 30 liters/minute.

Nerfilin I was purified from the supernatant by monitoring the induction activity of neurite outgrowth of PC12 cells. The supernatant collected by centrifugation was subjected to a Diaion HP-20 column, which was washed with 30% aqueous methanol and then eluted with 70% aqueous methanol. After evaporation, the active fraction was applied to an MPLC LiChroprep RP-18 (MERCK) column with 50% aqueous methanol. The active fraction was further purified by an HPLC TSKgel ODS-80TM (TOSOH) column with 50% aqueous methanol. Active compound nerfilin I and its derivative nerfilin II were concentrated to dryness separately to yield white powders of nerfilin I 3 mg and nerfilin II 100 mg from the total of 80 liters broth.

The physico-chemical properties of nerfilin I and nerfilin II are summarized in Table 1. Their molecular formulae were determined as $C_{28}H_{37}N_3O_5$ and $C_{28}H_{39}N_3O_5$, respectively, by HR-FAB mass spectroscopy using NBA matrix. The IR absorptions of nerfilin I and nerfilin II at 1635 cm^{-1} and 1540～1440 cm^{-1} suggested that these compounds are of a peptidic nature. In addition to these absorptions, nerfilin I showed a carbonyl group band at 1735 cm^{-1}.

The ^{1}H and ^{13}C NMR spectral data of nerfilin I and nerfilin II, summarized in Table 2 showed that they were similar to each other in structures. The ^{1}H NMR spectrum of nerfilin II in DMSO-d_6 proved the presence of 39 protons including 5 exchangeable ones. The carbons of nerfilin II were classified by ^{13}C NMR and DEPT spectral data as follows; $CH_3 \times 4$, $CH_2 \times 4$, $CH \times 5$, $CH= \times 9$, $C= \times 3$ and $C=O \times 3$. DQF-COSY, HMQC and HMBC experiments revealed the presence of three partial structures as shown in Fig. 1. Overlapping of two C=O carbon signals at δ_C 171.4 in the ^{13}C NMR spectrum of nerfilin II precluded the analyses of the long range correlations of NH protons to the C=O carbons. Amino acid analysis of the hydrolysate of nerfilin II indicated the presence of tyrosine and valine in nerfilin II. A FAB mass spectral fragment ion at m/z 251 was interpreted as caused by cleavage at the amide bond in Fig. 2. The absolute configurations of three α-CH carbons were determined to be all L through analyses of the hydrolysate of nerfilin II with HPLC chiral columns TSKgel ENANTIO L1 (TOSOH) and CROWNPAK CR (+) (DAICEL). Thus the structure of nerfilin II was elucidated as shown in Fig. 2. This structure was supported by good agreement of the NMR chemical shift values of nerfilin II with those of the relevant moieties in tyrostatin[3] (isovaleryl-tyrosil residue) and aurantimide[4] (N-acylphenylalaninol residue) shown in Fig. 3.

In the ^{13}C NMR spectra of nerfilin I in DMSO-d_6, a new signal appeared at δ_C 200.1 in place of the hydroxymethyl signal of the phenylalaninol group at δ_C 62.4 in the ^{13}C NMR spectrum of nerfilin II. The remaining signals of nerfilin I remained unchanged. The molecular formula of nerfilin I and its ^{13}C NMR signal at δ_C 200.1 suggested that the alcohol in nerfilin II was replaced with an aldehyde in nerfilin I as shown in Fig. 2. The NMR and FAB mass spectral data well supported the structure of nerfilin I. A fragment ion at m/z 249 of nerfilin I in the FAB mass spectrum was interpreted as caused by cleavage at the amide bond in Fig. 2.

The neurite outgrowth activity was measured as follows; PC12 cells were cultured in DULBECCO's modified Eagle medium (DMEM) containing 10% FCS until semiconfluent. The cells were trypsinised and re-suspended at a 1/4 concentration in DMEM containing 3% FCS and 50 ng/ml NGF and cultured for 24 hours. Then the cells were trypsinised and re-suspended in DMEM containing 1% FCS (24-well collagen-coated micro-plates, each well containing 400 μl). Test samples were added as methanol solutions and the activities were observed with a microscope after 24～48 hours.

Table 1. Physico-chemical properties of nerfilin I and nerfilin II.

	Nerfilin I	Nerfilin II
Appearance	White powder	White powder
MP	152～154°C	215～217°C (dec)
Molecular formula	$C_{28}H_{37}N_3O_5$	$C_{28}H_{39}N_3O_5$
HRFAB-MS (*m/z*)		
Found:	496.2817 $(M+H)^+$	498.2942 $(M+H)^+$
Calcd:	496.2813	498.2970
$[\alpha]_D^{24}$	−12.0° (*c* 0.025, CH_3OH)	−28.6° (*c* 0.028, CH_3OH)
UV λ_{max}^{MeOH} nm (ε)	260 (1,000), 269 (1,400), 278 (1,500)	259 (1,200), 269 (1,700), 279 (1,700)
IR ν_{max} (KBr) cm^{-1}	3280 (br), 2960～2850, 1735, 1635, 1540～1440, 1390, 1215	3270 (br), 2960～2870, 1635, 1540～1440, 1390, 1220

Table 2. NMR spectral data for nerfilin I and nerfilin II in DMSO-d_6.

Position	δ_C		δ_H	Position	δ_C		δ_H
	Nerfilin I	Nerfilin II	Nerfilin II		Nerfilin I	Nerfilin II	Nerfilin II
Isovaleryl				Valyl			
CH_3	22.1	22.1	0.70 (d, 7.0)	CH_3	17.7	18.0	0.77 (d, 6.2)
			0.74 (d, 7.0)		19.1	19.1	0.76 (d, 6.2)
CH	25.5	25.5	1.83 (m)	CH	30.7	30.8	1.88 (m)
CH_2	44.5	44.5	1.88 (d, 6.0)	α-CH	57.3	57.8	4.08 (dd, 8.8, 7.5)
C=O	171.5	171.4		C=O	171.5	170.2	
Tyrosyl				Phenylalaninol or Phenylalaninal			
NH			7.96 (d, 8.0)	NH			7.76 (d, 8.0)
α-CH	54.0	54.1	4.46 (m)	α-CH	59.7	52.2	3.91 (m)
β-CH_2	36.3	36.3	2.59 (dd, 13.0, 6.0)	β-CH_2	33.3	36.3	2.62 (dd, 13.0, 3.0)
			2.83 (dd, 13.2, 3.5)				2.86 (dd, 13.0, 5.0)
γ-C	128.2	128.0		γ-C	137.6	139.0	
δ-CH	130.0	129.9	7.02 (d, 8.0)	δ-CH	129.1	129.0	7.19 (m)
ε-CH	114.7	114.7	6.61 (d, 8.0)	ε-CH	128.2	128.0	7.21 (m)
ξ-C	155.6	155.7		ξ-CH	126.3	125.8	7.14 (m)
OH			9.15 (b)	CH_2		62.4	3.25 (dd, 10.0, 6.0)
C=O	171.5	171.4					3.32 (dd, 10.0, 5.0)
Valyl				OH			4.76 (b)
NH			7.68 (d, 8.8)	CHO	200.1		

Fig. 1. Partial structure of nerfilin II.

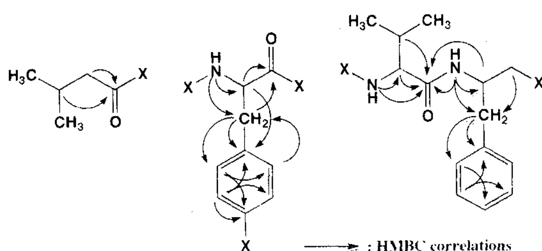


Fig. 2. Structures of nerfilin I and nerfilin II.

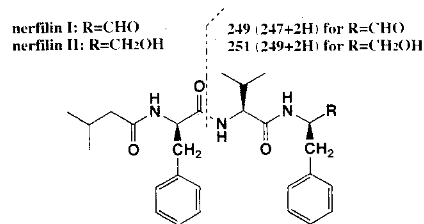
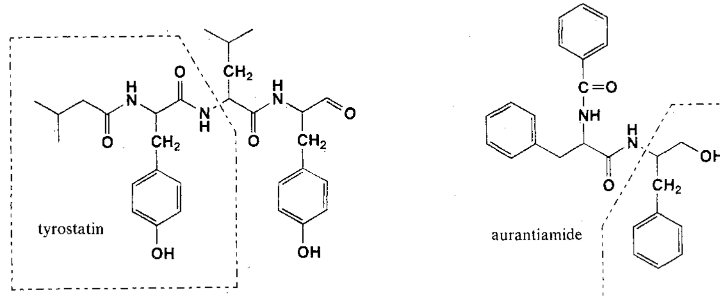


Fig. 3. Structures of tyrostatin and aurantiamide.



Nerfilin I induced neurite outgrowth of PC12 cells at concentrations of 0.5~0.05 μg/ml. At concentrations higher than 0.5 μg/ml, nerfilin I showed cytotoxicity against cultured PC12 cells. Nerfilin II did not induce the neurite outgrowth.

In addition, nerfilin I and nerfilin II inhibited papain at IC_{50} of 0.058 μg/ml and 0.10 μg/ml. Specific interactions between protease and some of protease-inhibitors have been suggested to play a role in the regulation of neurite outgrowth[5]. A leupeptin analogue, Ac-Leu-Leu-Nle-al was reported to stimulate the neurite outgrowth of PC12h cell in the presence of NGF[5]. Nerfilin I may induce the neurite outgrowth of PC12 by the same mechanism.

References

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