Stachybotrin C and Parvisporin, Novel Neuritogenic Compounds

I. Taxonomy, Isolation, Physico-chemical and Biological Properties

YURIKO NOZAWA, KYOKO YAMAMOTO, MAYUMI ITO, NORIYOSHI SAKAI, KAZUTOSHI MIZOUE, FUMIO MIZOBE and KAZUNORI HANADA

Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Omiya-shi, Saitama 330, Japan

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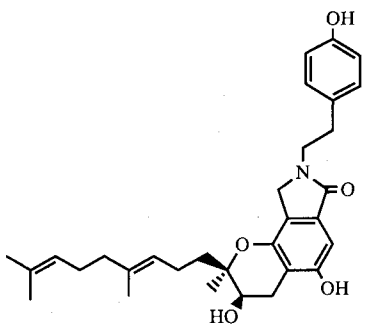
Stachybotrin C and parvisporin, novel neuritogenic compounds, were isolated from the culture broth of *Stachybotrys parvispora* F4708. Stachybotrin C induced significant neurite outgrowth in PC12 cells and showed cell survival activity in the primary culture of cerebral cortical neurons. Parvisporin demonstrated only weak neuritogenic activity.

Nerve growth factor (NGF), a polypeptide originally purified from mouse submaxillary gland, is the first and best characterized molecule to support the growth, differentiation and survival of neurons *in vitro*[1,2]. Survival and growth of cultured neuronal cells are also affected by such biological polypeptides as fibroblast growth factor. Epidemal growth factor, insulin and insulin-like growth factors are also known to target non-neuronal cells. These neurotrophic factors have been shown to protect against neuronal dysfunction and death *in vivo* in animal models of injury and neurologic disease. For example, NGF treatment was found to prevent the lesion-induced loss of septal cholinergic neurons in rats[3,4] and to reduce the occurrence of hippocampal delayed neuronal death following transient ischemia in Mongolian gerbils[5]. It was also reported that basic fibroblast growth factor (bFGF) prevents retrograde degeneration of thalamic neurons after cortical infarction in rats[6]. These observations rationalize the idea that low molecular compounds exhibiting neurotrophic actions can be developed as promising therapeutic drugs to prevent neuronal cell death occuring after cerebral hemorrhage caused by ischemia, and to ameliorate some types of dementia.

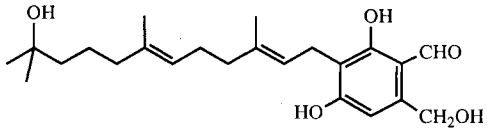
It is well known that the PC12 cell line, a rat pheochromocytoma, responds to NGF and bFGF by differentiating into neuron-like cells with elongated outgrowth[1,2,7]. In the present study, we have utilized, this property of PC12 cell line in the purification of novel neurotrophic compounds stachybotrin C and parvisporin from the culture broth of a *Stachybotrys parvispora* F4708. We have furthermore examined the effect of stachybotrin C and parvisporin on cell death in cultured rat cortical neurons by treatment with or without *in vitro* hypoxia.

This paper describes the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of stachybotrin C and parvisporin.

Fig. 1. Structures of stachybotrin C and parvisporin.



Stachybotrin C



Parvisporin

Materials and Methods

Taxonomic Studies

The strain F4708 was isolated from a leaf sample collected in Ohmiya-shi, Saitama Prefecture, Japan. For the evaluation of morphology and cultural characteristics, the strain was grown on potato glucose agar, oatmeal agar and malt extract agar.

Fermentation

A well grown slant culture of *Stachybotrys parvispora* F4708 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of seed medium consisting of glucose 2%, polypepton 0.5%, yeast extract 0.2%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, at pH 6.0. The seed culture was incubated at 26°C for 4 days on a rotary shaker (200 rpm). A 100 ml of the seed culture was transferred into a 5 liter jar fermenter containing 3 liters of production medium with the same composition as the seed medium. The fermentation was carried out for 4 days at 26°C under aeration of 1.0 v/v/minute and agitation of 300 rpm.

Assay for Neurite Outgrowth

PC12 cells were obtained from the RIKEN Cell Bank and grown in DULBECCO's modified EAGLE's medium (DMEM, GIBCO) with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS), 50 U/ml penicillin and 50 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Neurite outgrowth activity was evaluated mainly according to the same methods descrived by ITO *et al.*[8]. Briefly, the cells were plated on collagen-coated 24-well plates (Corning) at a density of 1×10^4 cells/well. After cultured for 24 hours, the medium was replaced with the medium containing a test compound, and the cells were further incubated for 48 hours. Cells were observed for scoring (round cells, 0; morphologically changed cells without neurite, 1; cells with neurites shorter than the diameter of the cell body, 2; cells with neurites longer than the diameter of the cell body, 3) under a phase-contrast microscope. One hundred cells were scored from a randomly chosen field and this was repeated 3 times (300 cells scored in total).

Neuronal Cell Preparation and Cell Survival Assay

The primary culture of cerebral cortical neurons was prepared from 18-day-old Wistar rat embryos. Dissociated neuronal cells in a 1 : 1 mixture of DMEM and HAM's F12 medium containing with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (DF-FBS medium) were plated on 24-well plates coated with polyethylenimine[9] at 1×10^5 cells/cm^2 and incubated at 37°C in a humidified 5% CO_2 incubator (day 0). On day 1, the medium was changed to a serum-free DF medium supplemented with 5 μg/ml transferin, 5 μg/ml insulin, and 20 μM progesterone (DF-TIP medium) in the presence of a test compound and further incubated for 5 days. On day 4, hypoxic stress was given by incubating the culture for 4 hours in a humidified atmosphere of 1% O_2 - 5% CO_2 in N_2, and these cells were cultured for a further 48 hours in 5% CO_2 in air. The effect of a test compound on the neuronal cells was evaluated by the activities of neuronal survival and neurite extention. Viable cells were measured by the MTT colorimetric method[10].

The neurite extention was monitored under a phase-contrast microscope in comparison with that of the control cells.

Results

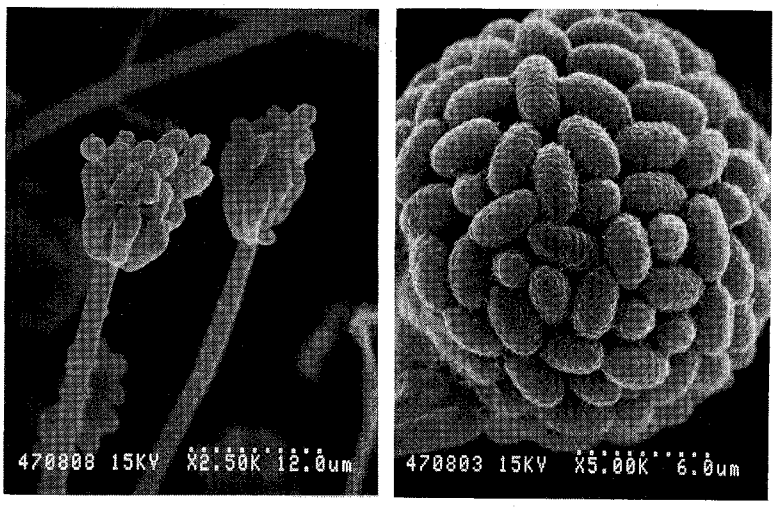
Taxonomy of the Producing Strain

For the cultural characterization, strain F4708 was incubated for 14 days at 26°C using three agar media described below.

Colonies on potato glucose agar grew moderately, attaining a diameter of 36~39 mm and were felty, dark yellowish gray in color. The reverse side of colonies was deep reddish yellow, and pale reddish yellow at the margin. Pale orange soluble pigments were produced into the agar. Colonies on oatmeal agar grew also moderately, attaining a diameter of 37~39 mm. They were felty to veltinouse, and very dark yellowish green in color. The rerverse side of colonies was pale yellow. No soluble pigment was observed, but clear exudate was abundantly present on the surface of agar. Colonies on malt extract agar grew rather slowly, attaining a diameter of 15~17 mm. They were felty and light greyish orange, and their reverse side was dull orange.

Morphological observations were made on the culture grown at 26°C on potato glucose agar. The scanning electron micrograph of *Stacybotrys parvispora* F4708 is shown in Fig. 2a. Conidiogenous cells were monophialidic. Conidiophores arose from aerial hypae or hyphal ropes, and mostly simple but sometime branched and were covered with granules. They were hyaline in color and smooth-walled, up to 83 μm long, and 2.9~4.8 μm thick at the base, tapering to 1.7~2.9 μm. Phialides were clavate, 6.7~11.4 μm long, and 2.9~4.8

Fig. 2. Scanning electron micrograph of *Stachybotrys parvispora* F4708.



(a) Bar represents 12.0μm (b) Bar represents 6.0μm

μm thick in the broadest part. Conidia were aggregated, unicellular, dark greyish green, ellipsoidal and $3.8 \sim 5.9 \times 2.7 \sim 2.9$ μm in size. The surface of conidia was smooth by a microscope, but a minutely roughened-wall was observed by a Scanning electron microscope (Fig. 2b).

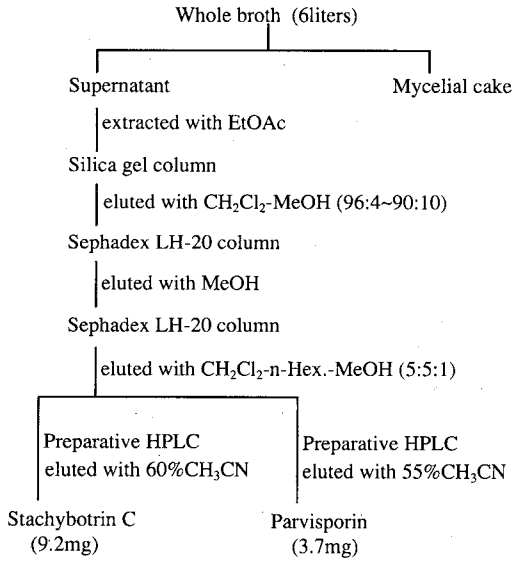
The results of morphology and cultural characteristics indicated that the strain F4708 was identified as one strain of *Stachybotrys parvispora* Hughes[11,12]. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-12660.

Isolation

Isolation of stachybotrin C and parvisporin are outlined in Fig. 3. Activity was monitored by the neurite outgrowth assay using PC12 cells.

The culture broth was filtered with the aid of filteraid to separate the mycelial cake and supernatant. The supernatant (5 liter, pH 5.1) was extracted 4 times with 2.5 liters of ethyl acetate, and the organic layer was evaporated under reduced pressure to yield a residue (2.7 g). This crude solid was chromatographed over a silica gel column using CH_2Cl_2 with increasing amounts of MeOH. The active fractions eluted with CH_2Cl_2-MeOH ($96:4 \sim 90:10$) were combined and evaporated to dryness to afford a light brown powder (386.5 mg). This sample was applied to a Sephadex LH-20 column and developed with MeOH. The active fractions were combined and concentrated. This material (110.6 mg) was then re-chromatographed over Sephadex LH-20

Fig. 3. Isolation procedure for stachybotrin C and parvisporin.



with CH_2Cl_2-*n*-hexane-MeOH (5 : 5 : 1) to separate two active compounds.

The active compound first eluted was further purified by preparative HPLC (column: Senshu-Pak ODS-4251-N, 10 i.d. × 250 mm) using 60% CH_3CN to give stachybotrin C (9.2 mg). The second active compound was subjected to preparative HPLC and eluted with 55% CH_3CN to yield parvisporin (3.7 mg).

Table 1. Physico-chemical properties of stachybotrin C and parvisporin.

		stachybotrin C	parvisporin
Nature		Yellowish white powder	Yellowish white powder
M.P.		89~92°C	73~75°C
$[\alpha]_D^{28}$ (c=0.1, MeOH)		-28.18°	+16.33°
Molecular formula		$C_{31}H_{39}NO_5$	$C_{23}H_{34}O_5$
HREI-MS	Observed	m/z 505.2840 (M^+)	
	Calcd	505.2828	
HRFAB-MS	Observed		m/z 389.2322 $(M-H)^-$
	Calcd		389.2328
UV λ_{max}^{MeOH} nm (ε)		216 (52800) 258 (12600) 301 (4000)	206 (16700) 295 (5800)
IR ν_{max}^{Neat} cm^{-1}		3305 3013 2971 2927 1662 1615 1516 1471	3368 2929 2856 1723 1621 1455

Fig. 4. ^1H NMR spectrum of stachybotrin C (400 MHz in $CDCl_3$).

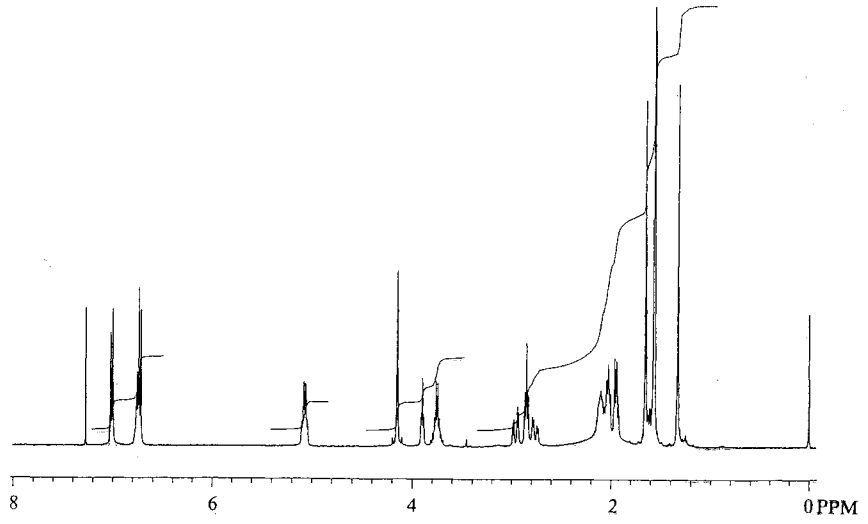
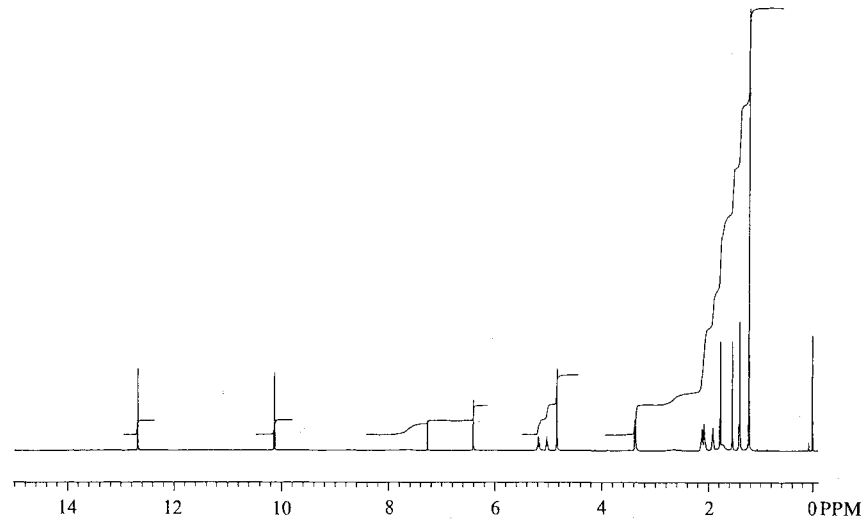


Fig. 5. ^1H NMR spectrum of parvisporin (400 MHz in $CDCl_3$).

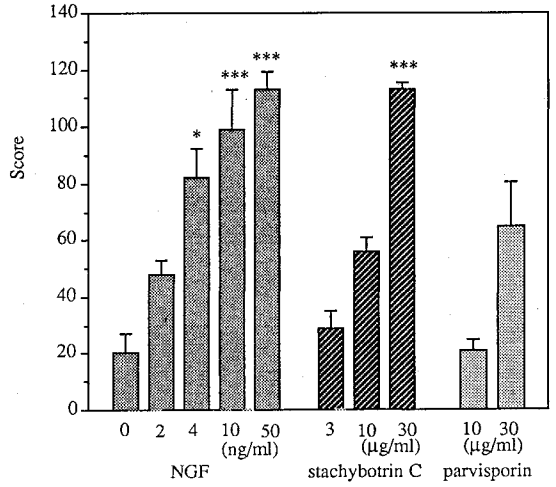


Physico-chemical Properties

Stachybotrin C and parvisporin were isolated as yellowish white powders. They are readily soluble in methanol, ethanol and acetone, slightly soluble in hexane and benzene and practically insoluble in water. They give positive color reactions to iodine, sulfuric acid, $FeCl_3$ but negative to ninhydrin.

The physico-chemical properties of stachybotrin C and parvisporin are summarized in Table 1. The IR and UV spectra of stachybotrin C are clearly different from those of parvisporin. Their molecular formulae are established to be $C_{31}H_{39}NO_5$ for stachybotrin C and $C_{23}H_{34}O_5$ for parvisporin, respectively, by their molecular ion (M^+) measurements in their HREIMS. Their ^1H NMR spectra are shown in Fig. 4 and 5 respectively. The structures of stachybotrin C and parvisporin are determined as shown in Fig. 1 by the extensive analyses of their NMR spectra and chemical derivatization. The details will be described in the succeeding paper[13].

Fig. 6. Effect of stachybotrin C and parvisporin on neurite outgrowth in PC12 cells.



Significantly different from control: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (t-text).

Biological Properties

Stachybotrin C and parvisporin generated neurite outgrowth PC12 as shown in Fig. 6. Stachybotrin C induced a significant neurite outgrowth at a dose of 30 μg/ml, corresponding to NGF at 50 ng/ml. Neurite induction activity of parvisporin was substantially weaker than that of stachybotrin C.

Cell survival activity of Stachybotrin C and parvisporin was examined using rat cortical neuronal cells. As shown in Table 2, stachybotrin C showed marked protective effects against neuronal cell damages. The maximal effect was observed at 3 μg/ml, and viable cells increased 7 fold in comparison with those of the control. This cell survival activity was also observed after a hypoxic stress condition, and the index of living neurons was almost the same as that in non-hypoxic condition. Furthermore, stachybotrin C generated neurite outgrowth in the primary neuronal culture (data not shown). Parvisporin showed no cell survival activity.

Discussion

Several neuritogenic compounds such as lactacystin[14], BU-4514N[15], PS-990[16], nerfilin I[17] and epolactaene[18], have been isolated so far from various microbial sources. Stachybotrin C is the first example of microbial metabolite which showed a protective effect in an *in vitro* anoxia model using a primary culture of cortical neurons. Since its protective effect on neuronal cell damage is pronounced, stachybotrin C is supposed to work as a neurotrophic factor in cerebral neurons, and is expected to prevent hypoxic neuronal injury caused by ischemia. Evaluation of stachybotrin C against a variety of experimental cerebral hypoxia and models of ischemia is underway.

Table 2. Effect of stachybotrin C and parvisporin on cell survival in primary neuronal culture.

		neuronal survival			
		hypoxic-stress		non-treatment	
Test compound	Dose (μg/ml)	OD515	% of control	OD515	% of control
control		0.141	100	0.136	100
stachybotrin C	0.3	0.220	156	0.233	171
	1	0.282	200	0.337	247
	3	0.951	674 **	0.949	697 **
parvisporin	0.3	0.141	100	0.314	231
	1	0.238	169	0.280	206
	3	0.331	235	0.290	213

** : neurite outgrowth was observed

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