PS-990, A NOVEL NEURITOGENIC COMPOUND FROM *Acremonium* sp.

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A novel compound, PS-990, which induces differentiation of neuroblastoma cells, was isolated from the culture broth of a fungus, *Acremonuim* sp. KY12702. PS-990 inhibited brain calcium/calmodulin-dependent cyclic nucleotide phosphodiesterase with an IC_{50} value of 3 μg/ml, and markedly induced neurite extension of mouse neuroblastoma, Neuro2A, at concentrations ranging from 10 to 30 μg/ml.

Neurotrophins are thought to be able to affect survival, target innervation and/or function of neuronal cell population. Evidence has been accumulated to show that these molecules might have the potential to become pharmaceutical agents in pathological conditions affecting both the central and the peripheral nervous system[1,2].

Cyclic AMP analogs can replace nerve growth factor in promoting long-term survival and neurite outgrowth in cultured sympathetic and sensory neurons[3]. Recently, there are some papers showing a positive relationship between neurite formation and the intracellular cyclic AMP concentration. Cyclic nucleotide phosphodiesterase (PDE) inhibitors, griseolic acid[4] or KS-505a[5], stimulate the neurite outgrowth along with the elevation of the intracellular cyclic AMP level in Neuro 2A cells. These compounds which exhibit neurite extension would be useful in the treatment of various neurodegenerative diseases including senile dementia.

In the course of our screening work to obtain PDE inhibitors which possess neuritogenic activities from a microbial source, we found that a novel metabolite of *Acremonium* sp. KY12702 inhibited calcium/calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE), and stimulated neurite extension in a murine neuroblastoma cell, Neuro2A. The compound was designated PS-990. In this article, we report production, isolation and some biological properties of PS-990.

Materials and Methods

Materials

The murine neuroblastoma cell lines, Neuro2A were obtained from American Type Culture Collection. Culture media were purchased from Nissui Pharmaceutical (Tokyo, Japan), and fetal calf serum was from JRH Biosciences (Lenexa, KS, U.S.A.). Non-essential amino acid solution was purchased from Flow Laboratories (Irvine, Scotland, U.K.). Dibutyryl cyclic AMP was obtained from Sigma (St. Louis, MO, U.S.A.). Bovine brain calcium/calmodulin-dependent PDE (CaM-PDE), bovine heart calmodulin-independent cyclic-nucleotide phosphodiesterase (CaM-independent PDE), and calmodulin (CaM) were prepared according to the method of KAKIUCHI[6] *et al.* with some modifications[7]. All other chemicals were analytical grade.

Microorganism

The producing microorganism, *Acremonium* sp. KY12702 (FERM BP-4019) was isolated from a soil collected in Tokyo, Japan.

Culture and Medium Conditions

A loopful spores of microorganism, grown on an agar slant, was inoculated into 10 ml of seed medium composed of V8 vegetable juice (Cámpbell) 20% and $CaCO_3$ 0.3% (pH 6.4 before sterilization) in test tube (21 i.d. × 200 mm). The agar slant medium consisted of malt extract 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar 2% (pH 6.5 before sterilization). The inoculated tube was incubated at 25°C for 5 days. A 10%-inoculation from the above vegetative medium was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. After cultivation for 2 days on a rotary shaker (200 rpm) at 25°C, 50 ml of the second seed culture was transferred to 2-liter Erlenmeyer flask containing 500 ml of the fermentation medium composed of glucose 2%, dried mashed potato (Yukijirushi) 2%, peptone (Kyokuto) 0.5%, K_2HPO_4 0.05% and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 6.0 before sterilization) and incubated for 7 days at 25°C on a rotary shaker (200 rpm). The growth was monitored by packed cell volume (PCV) measurement. The PS-990 was produced in mycelia, and its production was traced by HPLC. For this measurement, 5 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 5 ml of methanol. The extract was passed through the Sep-Pak C18 (Waters), and 10 ~ 20 μl of the sample was provided for HPLC assay.

Determination of PS-990 by HPLC

PS-990 produced was determined by an HPLC system equipped with a Shimadzu LC9A pump, Shimadzu SPD-6A UV detector and Shimadzu C-R4AX Chromatopac. PS-990 extracted form mycelia was injected onto an octadecylated silica gel column (AQ-312, 6 i.d. × 150 mm, YMC) and developed with 60% acetonitrile solution at a flow rate of 1 ml/minute at 40°C, monitoring absorbance at 220 nm.

Cell Culture

Neuro 2A cells were cultured in minimum essential medium containing 10% fetal calf serum and 1% nonessential amino acid solution. Cultures were maintained at 37°C under a humidified atmosphere of 5% CO_2 in air. Cells were detached from the plates by treatment with 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.1 mM EDTA. The cells were distributed in 12-well plate (Corning) at an inoculum of 1.2×10^5 cells/well, followed by incubation for 24 ~ 48 hours. After removal of the medium, 1 ml of fresh medium with or without a compound was added. PS-990 was dissolved in methanol or dimethylsulfoxide, and dibutyryl cyclic AMP was in PBS. The control cultures received the appropriate amounts of the solvents, methanol and dimethylsulfoxide (0.2 ~ 1%). At these concentration of the solvents themselves had no effect on the neuroblastoma cells.

Measurement of Neurite Formation

After cells were cultured for 12 hours, photomicrographs of randomly chosen field were taken. Neurite formation was measured as described by GUZWITS and CUNNINGHAM[8]. In brief, cells exhibiting at least one clearly defined neurite equal to or longer than one cell diameter were scored as positive.

Measurement of PDE Activity

The activity of PDE was measured by HPLC. The reaction mixture contained in a final volume of 0.5 ml, 80 mM imidazole-HCl buffer (pH 6.9), 3 mM $MgSO_4$, 0.3 mM dithiothreitol, 100 mM NaCl, 1.2 mM cyclic AMP, 50 μM $CaCl_2$, varied concentrations of PS-990, 10 mU/ml bovine brain CaM-PDE and 4 U/ml CaM. The activity of CaM-independent PDE was determined in the presence of 20 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid instead of Ca^{2+}/CaM. The reaction mixture was incubated at 28°C for 30 minutes. After which, the reaction was terminated by the addition of 200 μl of 10% acetic acid. 10 μl of the reaction mixture was injected onto octadecylated silica gel column (YMC ODS AQ-312, 6 i.d. × 150 mm, YMC) and developed with 7.5% acetonitrile solution containing 0.1% acetic acid at a flow rate of 1 ml/minute at room temperature, monitoring absorbance at 260 nm. The product of the enzyme reaction, 5'-AMP, was eluted at approximately 3.5 minutes after the injection.

Results

Taxonomy

The fungal strain KY12702 was isolated from a soil sample collected in Tokyo, Japan. Colonies on 2% malt extract agar medium were *ca.* 30 mm in diameter after culturing at 25°C for 14 days. The surface of a colony was white and the reverse was cream and pale brown at the center. On 2% malt extract agar medium, vegetative hyphae were thin-walled, smooth and septate. Phialides were mostly simple, hyaline, smooth, 11.5~20 μm long, 0.7~2 μm wide at the base, tapering towards the tip (*ca.* 0.3 μm wide). Conidia were nonseptate, hyaline, smooth-walled, ellipsoidal, long-ellipsoidal, fusiform, clavate, reniform, and sickle-shaped, 1.5~7×0.7~1.8 μm, aggregated into spherical heads.

From the characteristics mentioned above, the fungus strain KY12702 was identified as *Acremonium* sp.[9].

Production of PS-990 by Fermentation

The time course of PS-990 production in 2-liter flask is shown in Fig. 1. The production of PS-990 in the culture broth initiated on day 3 and the amount of PS-990 was reached maximum on day 6. The amount of PS-990 produced in mycelia is approximately 10-fold higher than that in broth filtrate.

Isolation and Purification

The isolation procedure for PS-990 is outlined in Fig. 2. PS-990 was mainly purified from mycelia obtained by filtration of the fermentation broth. The mycelial cake was extracted with methanol. The extract was concentrated *in vacuo* to give aqueous solution which was then extracted with ethylacetate. The organic layer was concentrated *in vacuo*, to yield an oily material, which was dissolved in a small volume of chloroform and applied to a silica gel

Fig. 1. Time course of PS-990 production in a 2-liter Erlenmeyer flask.

● PS-990, ○ growth (PCV), △ pH.

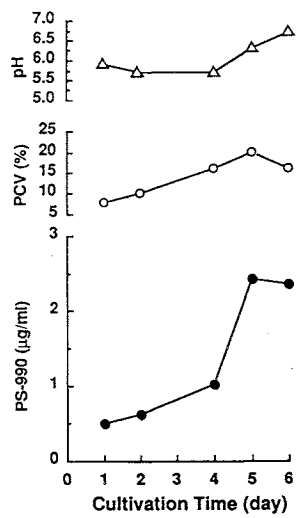
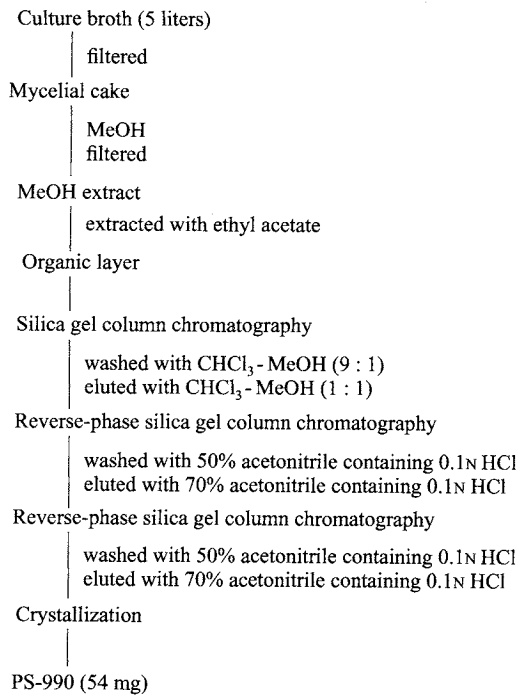


Fig. 2. Purification of PS-990.



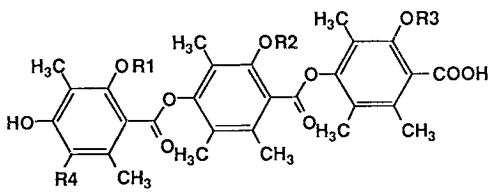
column (Merck Art. 7734, 700 ml). The column was washed with chloroform - methanol (9 : 1), and adsorbed material was eluted with chloroform - methanol (1 : 1). Fraction containing PS-990 were pooled and concentrated *in vacuo* to yield crude PS-990. The crude PS-990 was applied to a reverse-phase silica gel column (YMC ODS-A 60-230/70, 60 ml). The column was washed with 50% acetonitrile solution containing 0.1 N HCl, and adsorbed material was eluted with 70% acetonitrile containing 0.1 N HCl. Active fractions were pooled and concentrated *in vacuo* to dryness to yield yellow solid. The yellow solid was dissolved in 50% acetonitrile containing 0.1 N HCl, and applied to a reverse-phase silica gel column (YMC ODS-AM 120-400/230, 100 ml). The column was washed with 50% acetonitrile containing 0.1 N HCl, and adsorbed material was eluted with 70% acetonitril containing 0.1 N HCl. Fractions containing PS-990 thus obtained was dissolved in methanol and allowed to stand for one day at 4°C to yield colorless crystalline PS-990 (54 mg).

Table 1. Physico-chemical properties of PS-990.

Appearance	Colorless crystalline
Molecular formula	$C_{32}H_{36}O_{10}$
EI-MS	579.2230
MP (°C)	183~185
IR (KBr) cm^{-1}	3452, 2931, 1734, 1716, 1701, 1662, 1576, 1464, 1313, 1151, 1095, 1074
UV λ_{max}^{MeOH} nm (ε)	215, 257, 323
TLC (Rf)	
$CHCl_3$ - MeOH (9 : 1)[a]	0.08
$CHCl_3$ - MeOH - EtOH - H_2O[a] (10 : 4 : 4 : 2)	0.51
70% MeOH[b]	0.14
Color reaction	
Positive	I_2, H_2SO_4, Anisaldehyde
Negative	Ninhydrin, Rodon-Smith
Solubility	
Soluble	MeOH, acetone, DMSO, $CHCl_3$, EtOAc
Insoluble	*n*-Hexane

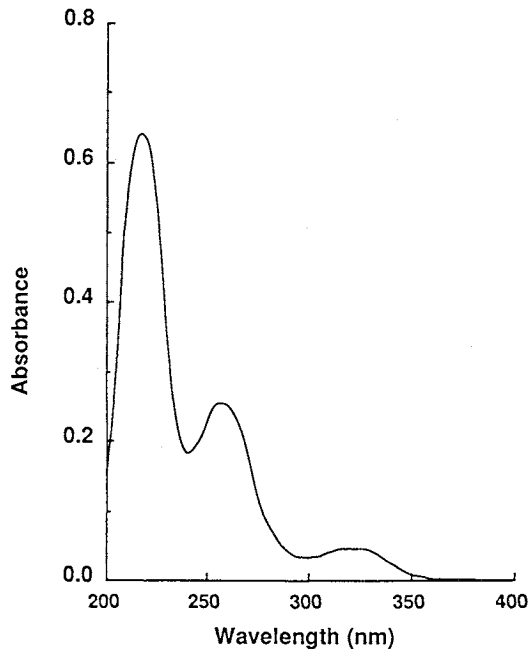
[a] Silica gel $60F_{254}$ plate (Merck Art. 5628).
[b] RP-$18F_{254}$S plate (Merck Art. 13724).

Fig. 4. The structures of PS-990 and thielavins.



Thielavin A $R_1=H$, $R_2=H$, $R_3=H$, $R_4=H$
B $R_1=H$, $R_2=CH_3$, $R_3=CH_3$, $R_4=H$
C $R_1=H$, $R_2=CH_3$, $R_3=CH_3$, $R_4=CH_3$
PS-990 $R_1=CH_3$, $R_2=H$, $R_3=CH_3$, $R_4=CH_3$

Fig. 3. The UV spectrum of PS-990.



The UV spectrum of PS-990 was obtained by using Hitachi spectrophotometer. 220A. PS-990 was dissolved in MeOH (10 μg/ml) and the spectrum was scanned through 1 cm of light path.

Table 2. The inhibitory activities of PS-990 for various PDEs.

Enzyme	IC_{50} (μg/ml)
Bovine brain CaM-PDE	
Native	3
Trypsin-digested	3
Bovine heart CaM-Independent PDE	38

Physico-chemical Properties of PS-990

Physico-chemical properties of PS-990 are summarized in Table 1. PS-990 is readily soluble in methanol, acetone and dimethylsulfoxide, soluble in chloroform and ethyl acetate, and virtually insoluble in *n*-hexane. The molecular formula of PS-990 was determined to be $C_{32}H_{36}O_{10}$ on the basis of high-resolution electron impact mass spectrum (EI-MS). The UV spectra of PS-990 are shown in Fig. 3. The structure of PS-990 was determined as shown in Fig. 4, on the basis of ^{1}H and ^{13}C NMR spectrum data. The methyl ether structure of PS-990 may not be formed during isolation because PS-990 was obtained by extraction from mycelia with 40% *n*-propanol instead of methanol.

Biochemical Properties

PS-990 inhibited bovine brain CaM-PDE with an IC_{50} value of 3 μg/ml, whereas CaM-independent PDE was inhibited at higher concentrations of PS-990 (Table 2). The IC_{50} value for CaM-PDE was not affected by CaM or calcium concentration (Fig. 5). The catalytic domain generated by tryptic digestion

Fig. 5. Inhibition by PS-990 of bovine brain CaM-PDE.

CaM-PDE activity was measured as described in Materials and Methods in the presence of (a) 4 (○), 8 (●), 16 (□), 32 (■) U/ml of CaM or (b) 25 (○), 50 (●), 100 (□) μM of calcium. All the experiments were carried out in duplicate.

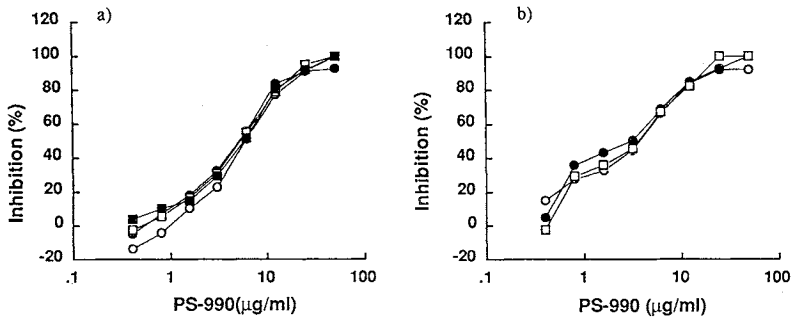


Fig. 6. Kinetic analysis of inhibition of CaM-PDE by PS-990.

The enzyme activity was assayed with cyclic AMP as variable substrate in the absence (○) or in the presence of 2.5 (●), 0.27 (■) μg/ml of PS-990. All the experiments were performed in duplicate.

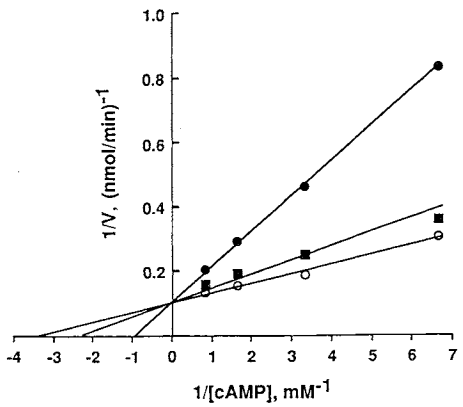
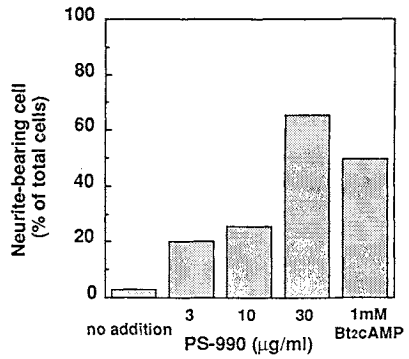


Fig. 7. Neuritogenic effect of PS-990.



The number of neurite-bearing cells were counted as described in Materials and Methods. Bt_2cAMP indicates dibutyryl cyclic AMP. All the experiments were performed in triplicate.

of CaM-PDE was inhibited by PS-990 with an IC_{50} value similar to that of the native enzyme (3 μg/ml). Double-reciprocal plot of cyclic AMP concentration versus reaction rate showed that the inhibition was competitive with respect to cyclic AMP (Fig. 6).

PS-990 was added to the culture medium of a neurally derived cell line, Neuro2A. The control Neuro 2A cell cultured without drugs extended quite a few neurites. When the cells were treated with PS-990 at concentrations ranging from 10 to 30 μg/ml, many neurites were extended from the cell bodies in a dose-dependent manner (Fig. 7). At concentrations higher than 100 μg/ml, PS-990 exerted its cytotoxic effect such as detachment of cells. Neurite formation in neuro2A cells was also observed in the presence of 1 mM dibutyryl cyclic AMP.

Table 3. The antimicrobial activities of PS-990.

Test microorganisms	MIC (μg/ml)
Candida albicans ATCC 10231	>83
Staphylococcus aureus ATCC 6538P	10
Pseudomonas aeruginosa BMH No. 1	>83
Enterococcus faecium ATCC 10541	21
Escherichia coli ATCC 26	>83
Bacillus subtilis No. 10707	0.65
Proteus vulgaris ATCC 6897	>83
Shigella sonnei ATCC 9290	>83
Klebsiella pneumoniae ATCC 10031	>83

The antimicrobial activity of PS-990 is shown in Table 3. PS-990 has activity against *Bacillus subtilis*, and weak activity against *Enterococcus faecium* and *Staphylococcus aureus*. The LD_{50} value of PS-990 was higher than 300 mg/kg in rats by po administration.

Discussion

In this paper, we demonstrate that a novel compound, PS-990, isolated from *Acremonium* sp., induces neurite elongation of a mouse neurally derived cell line, Neuro2A. Thielavins (Fig. 4), which is a related compound of PS-990, have been previously reported as inhibitors of biosynthesis of prostaglandins isolated from the cultured broth of *Thielavia terricola* SANK 15876[10,11]. Recently, microbial compounds whose structures closely resemble that of PS-990 have also been obtained from *Thielavia terricola* RF-143 as inhibitors of phospholipase A_2[12~14]. However, it remains unknown whether or not these compounds have inhibitory potency for PDE or neuritogenic activity.

PS-990 equally inhibited both intact CaM-PDE and partial tryptic digestion of the CaM-PDE which is insensitive to CaM. Inhibition of CaM-PDE by PS-990 was not recovered by higher concentration of CaM or calcium. Moreover, the mode of inhibition was competitive with respect to cyclic AMP. These results suggest that PS-990 may interact with the cyclic AMP-binding site in the catalytic domain of CaM-PDE.

It has been reported that neurite extension in primary cultured neurons or various neuronal cell lines was elicited by the agents such as cyclic AMP analogues[3], lactacystin[15], KS-505a[5], and griseolic acid[4], which elevate the intracellular cyclic AMP concentration. Both KS-505a and griseolic acid are inhibitors of cyclic nucleotide phosphodiesterase which catalyzes the degradation of cyclic AMP. PS-990 also inhibits cyclic nucleotide phosphodiesterase, however, the precise mechanism of PS-990-induced neuritogenesis still remains unclear. Further investigations are being now undertaken.

Finally, PS-990 may be a useful tool to understand the mechanism of neurite formation of neuronal cells. It may possess neuroprotective properties that are useful in the treatment of diseases involving the dysfunction of nervous system due to deficiency of neurotrophic factor and also in the study of medication of neuroblastoma.

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