S19159, a Modulator of Neurite Outgrowth Produced by the Ascomycete *Preussia aemulans*

I. Producing Strain, Fermentation, Isolation and Biological Activity

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A modulator of neurite outgrowth, designated S19159, was isolated from the fermentation broth of fungal strain 19159. This fungus was identified as the loculoascomycete, *Preussia aemulans* (Rehm) von Arx. In the presence of S19159, the number of neurites extending from the cell bodies of cerebral cortical neurons was markedly reduced. The effect of S19159 was observed specifically in neurons from the central nervous system. The compound exhibited similar activities on cultured cortical, hippocampal and cerebeller neurons but was without detectable effect on dorsal root ganglion neurons and PC12 cells.

Neuronal cells extend their processes along appropriate pathways to find their correct targets during development. Secreted and cell surface ligands in the surrounding environment control these events[1]. However, how these extracellular signals are transduced and processed within the growing axons is poorly understood, particularly in the growth cone. Therefore, a substance that modulates neurite outgrowth may be useful in analyzing the mechanism of neurite outgrowth.

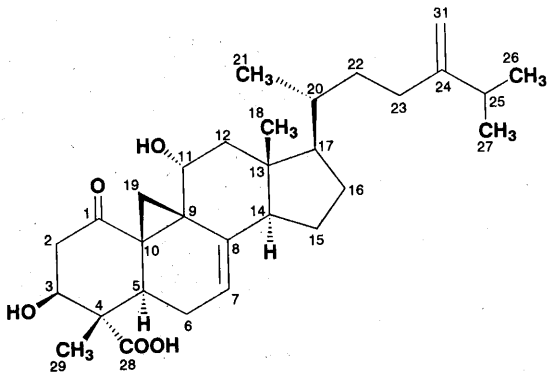
In the course of a screening program for substances that modulate neurite outgrowth from cerebral cortical neurons, S19159 was discovered in the fermentation broth of fungal strain 19159 identified as *Preussia aemulans*. The structure of S19159 is shown in Fig. 1. The details of the structure elucidation will be reported in a separate paper[2]. In this paper, the producing strain, fermentation, isolation, and biological activity of S19159 are described.

Materials and Methods

Microbe and Cultural Conditions

The producing strain 19159 was isolated from a leaf sample collected in Tateyama, Toyama Prefecture, Japan. For the observation of morphological characteristics, the producing strain 19159 was cultured on

Fig. 1. Structure of S19159.



oatmeal agar medium at 25°C and was observed under a light microscope. The color descriptions were based on Methuen Handbook of Colour[3].

Cell Culture

Cerebral cortex was dissected from embryonic day 18 rats and digested with papain. Tissue was washed in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS) and 5% horse serum (HS), and then mechanically dissociated by pipetting. Obtained cells were cultured in DMEM supplemented with 5% FCS, 5% HS, 5 μg/ml insulin, 5 μg/ml transferrin and 1 mM sodium pyruvate in a humidified 10% CO_2 atmosphere. For the assay of neurite outgrowth, cells were plated into 96-well plate coated with polyethyleneimine at the cell density of 1.24×10^5 cells/cm^2 in the medium containing various screening samples. The morphology of cells was observed after 24 or 48 hours. For the quantitation of neurite number, neurites that were longer than the cell diameter were counted for individual cells. More than 100 cells were counted in eight randomly chosen fields.

Instruments

FAB mass spectra were recorded on a JEOL JMS HX-110 mass spectrometer using *m*-nitrobenzyl alcohol as a matrix and polyethylene glycol as an internal standard for HR FAB-MS. ^{1}H and ^{13}C NMR spectra were taken with a JEOL A-600 FT NMR spectrometer using TMS as an internal standard. The UV spectrum was measured with a Shimadzu UV-240 spectrophotometer. The melting point was determined with a Yanagimoto micro melting point apparatus and is uncorrected.

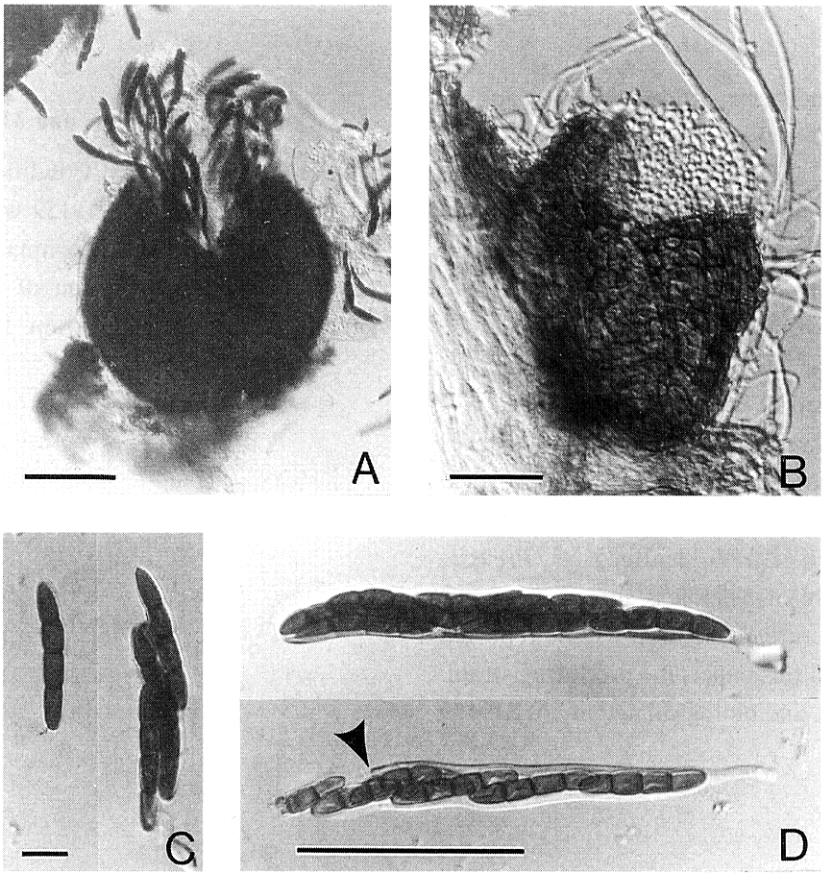
Results

Identification of Producing Strain 19159

Colonies of the fungal strain 19159 were 40~44 mm in diameter after culturing at 25°C for 14 days on oatmeal agar medium. The surface of the colony was floccose, greyish yellow (2B5), and the reverse was greyish

Fig. 2. Morphological characteristics of *Preussia aemulans* strain 19159.

A; Ascoma, bar represents 100 μm. B; *Phoma*-like pycnidium, bar represents 20 μm. C; Ascospores, bar represents 10 μm. D; Bitunicate asci, arrowhead indicates the tip of the ruptured ectotunica (outer ascus wall), bar represents 50 μm.



Magenta (13D5). On oatmeal agar medium, many ascomata and pycnidia were produced. Ascomata were dark brown, globose, non-ostiolate and 260~480 μm in diameter. Asci were cylindrical, bitunicate (inconspicuous), 8-spored and 108~148 × 10~13 μm. Ascospores were 4-celled, moderately constricted at the septa, dark brown, thick-walled, each cell with a longitudinal or oblique germ slit, 28~36 × 3.7~5.3 μm and broadly rounded at terminals. *Phoma*-like pycnidia were 47~103 μm in diameter and conidia were subglobose, hyaline and 2.0~2.7 × 1.5~1.9 μm. Morphological characteristics are shown in Fig. 2.

The fungal strain 19159 agreed well with the descriptions provide by VON ARX and STORM[4], VON ARX[5], DOMSCH *et al.*[6] and also with the subcultures of CBS 120.66 (=IAM 14571: derived from the type) and CBS 287.67 (=IAM 14572) of *Preussia aemulans* (Rehm) von Arx, morphologically as well as culturally. A subculture of the strain 19159 has been deposited in the IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, as IAM 14730.

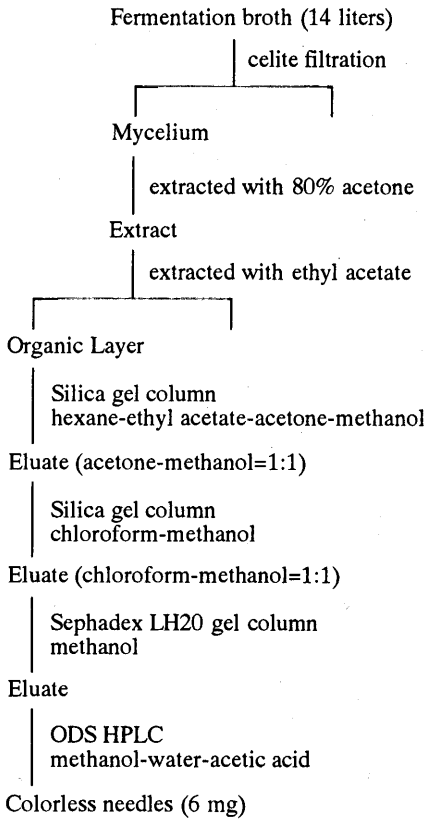
Fermentation

A slant culture of strain 19159, grown on malt-yeast-extract agar, was inoculated into 500-ml flasks containing 100 ml of a seed medium consisting of saccharose 4%, cotton seed meal 2%, dry yeast 1%, polypeptone 1%, KH_2PO_4 0.2%, $CaCO_3$ 0.2% and Tween 80 0.1%. The flasks were incubated on rotary shaker (120 rpm) at 25°C for 5 days. The seed culture was transferred at 1% to a 30-liter jar-fermenter containing 15 liters of a medium consisting of soluble starch 3%, peanut powder 1%, soybean meal 1%, KH_2PO_4 1% and Na_2HPO_4 4%. The fermentation was carried out at 25°C for 5 days employing aeration at 15 liters/minute and agitation at 300 rpm.

Isolation

Isolation procedures for S19159 are summarized in Fig. 3. The culture broth (14 liters) was filtered and the mycelium was extracted with 14 liters of 80% aqueous acetone. The extract was concentrated to 4.5 liters, and extracted with 9 liters of ethyl acetate. The organic layer was separated, dried with anhydrous magnesium sulfate and concentrated to dryness. The oily residue was applied on a column (500 ml) of silica gel (Silica gel 60 MERCK), which was washed with hexane, ethyl acetate and ethyl acetate-acetone (1:1). The active material was eluted with acetone-methanol (1:1). The eluate was further applied on a column (150 ml) of silica gel and developed stepwise with chloroform-methanol. The active fraction, chloroform-methanol (1:1), was concentrated, applied on a Sephadex LH20 column (25 i.d. × 700 mm) and eluted with methanol. The crude material was purified by HPLC (CAPCELL PAK C18: 4.6 i.d. × 250 mm, mobile phase: 90% aqueous methanol, 0.1% acetic acid). The active peak was collected and concentrated to give colorless needles (6 mg).

Fig. 3. Isolation scheme for S19159.



Physico-chemical Properties

The physico-chemical properties of S19159 are summarized in Table 1. High resolution FAB-MS measurement indicated the molecular formula of S19159 to be $C_{30}H_{44}O_5$. The UV spectrum (Fig. 4) exhibited a maximum at 205 nm with shoulders at 240 and 275 nm. The ^1H and ^{13}C NMR spectra are shown in Figs. 5 and 6. S19159 is soluble in methanol and ethanol and insoluble in water and hexane. S19159 gave positive color reactions to 2,4-dinitrophenylhydrazine and potassium permanganate tests but was negative to aniline-phthalate reagent.

Effect of S19159 on Neurite Outgrowth

As shown in Fig. 7, the effect of S19159 on cerebral

Table 1. Physico-chemical properties of S19159.

Appearance	Colorless needles
MP (°C)	>196 (decomp.)
Molecular weight	484
Molecular formula	$C_{30}H_{44}O_5$
FAB-MS (*m/z*)	485 $(M+H)^+$
HR FAB-MS (*m/z*)	
Found:	485.3256
Calcd:	485.3267 (for $C_{30}H_{45}O_5$)
UV λ_{max}^{MeOH} nm (ε)	205 (11,180), 240 (sh, 2,180), 275 (sh, 530)
Solubility	
Soluble:	MeOH, EtOH
Insoluble:	H_2O, hexane

Fig. 4. UV spectrum of S19159 (MeOH).

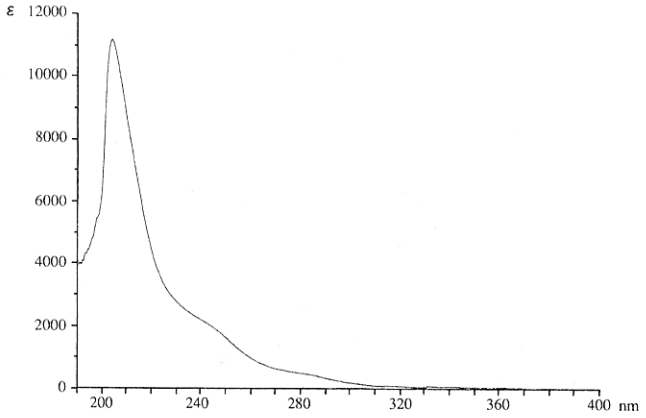


Fig. 5. ^1H NMR spectrum of S19159 (600 MHz, CD_3OD).

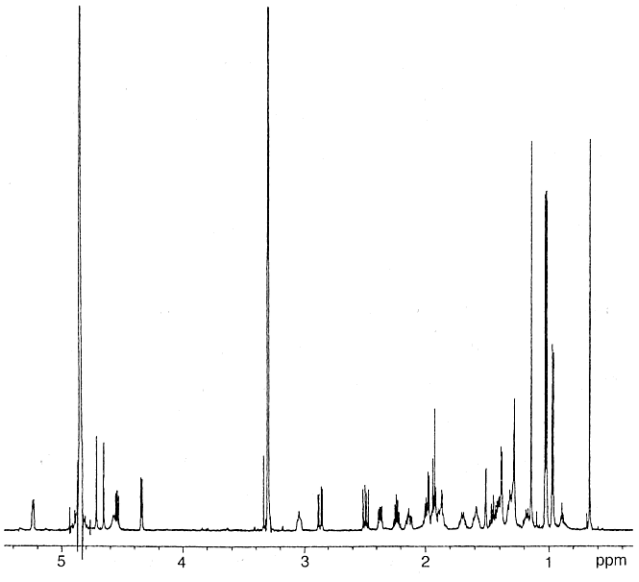


Fig. 6. ^{13}C NMR spectrum of S19159 (150 MHz, CD_3OD).

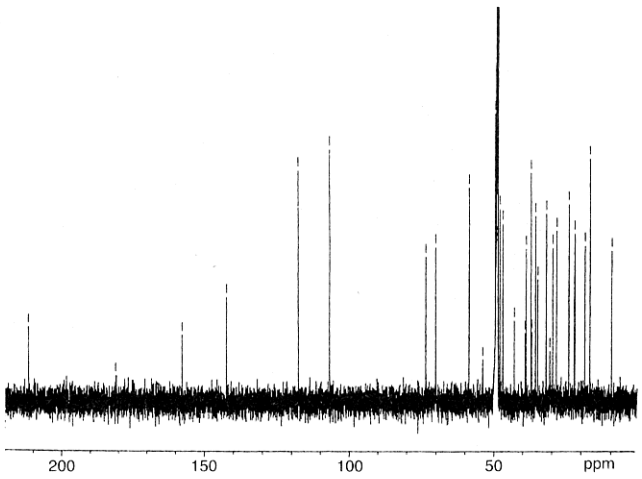
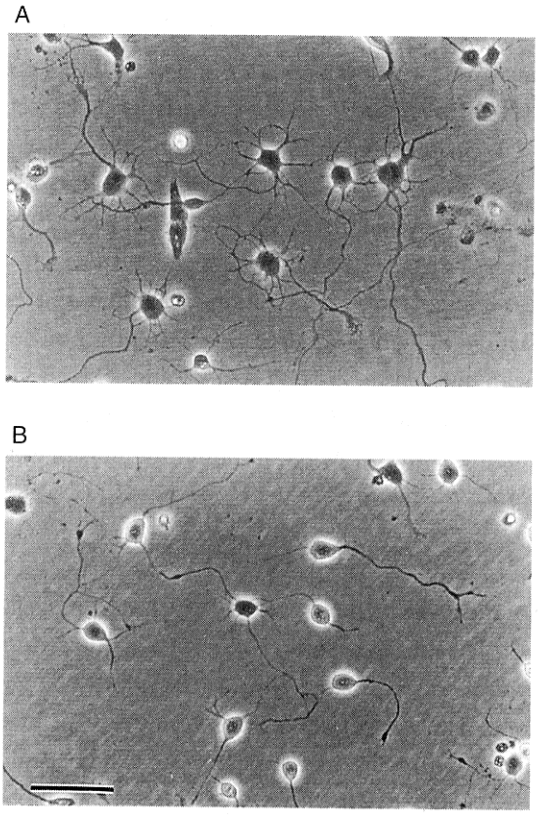


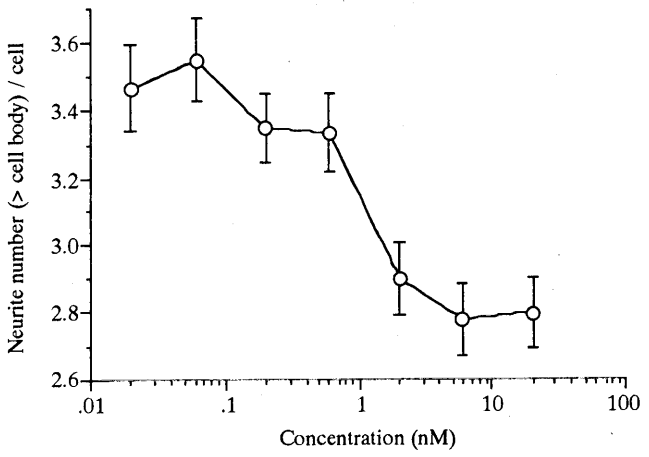
Fig. 7. Effect of S19159 on the morphology of cerebral cortical neurons.

Cerebral cortical neurons cultured for 48 hours. A: Control, B: S19159 20 nM, bar represents 50 μm.



cortical neurons was examined. Cerebral cortical neurons grown in culture have long and well branched neurites (Fig. 7A). In contrast, in the presence of S19159, the number of neurites extending from the cell body was

Fig. 8. Effect of S19159 on neurite number extending from the cell body.



reduced and these neurites were less branched. The morphology of the cell bodies was rounded and refractive, indicating that they are less attached to the substrate (Fig. 7B). The effective concentration of S19159 on neurite number is indicated in Fig. 8. Similar effects were observed in rat hippocampal and cerebeller neurons. However, S19159 exhibited little effect on neurite outgrowth from rat pheochromocytoma cell line PC12-22a and rat dosal root ganglion neurons stimulated with nerve growth factor, even at the highest concentration examined ($>20\,\mu$M; data not shown). Thus, the effect of S19159 on the morphology of extending neurites is likely to be specific to neurons derived from the central nervous system (CNS).

Discussion

Although numerous extracellular and cell-surface molecules involeved in neurite formation have been identified to date, underlying intracellular mechanisms are largely unknown. We seached for a substance that modulates neurite outgrowth from cerebral cortical neurons and discovered S19159. Since the action of S19159 is specific to neurons from the CNS, this compound may be useful as a biological probe in analyzing the mechanism of neurite outgrowth from CNS neurons. The structure of S19159 is related to some oxygenated sterols that exhibit many biological activities including inhibition of cholesterol biosynthesis, cytotoxic effects, carcinogenic activities and immunological effects[7,8]. Indeed, the rapid and extensive morphological and cytotoxic effects of S19159 were apparent in cerebral cortical neurons cultured in lipid-deficient medium which is almost devoid of cholesterol. These could be partially reversed by the addition of choresterol to the culture medium, but never completely reversed. Further work is needed to examine the action of S19159.

Acknowledgments

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