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A Novel Neuritogenic Compound, NGA0187

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A new neuritogenic compound NGA0187 was isolated from the fermentation broth of *Acremonium* sp. TF-0356. The structure of NGA0187 was determined by means of spectroscopic analysis and X-Ray diffraction. NGA0187 induced significant neurite outgrowth in PC12 cells. However, survival effect of NGA0187 on the primary culture of cerebral cortical neurons was not observed.

Neurotrophic factors have been shown to protect against neuronal dysfunction and death *in vivo* in animal models of injury and neurologic disease. For example, nerve growth factor (NGF) treatment was found to prevent the lesion-induced loss of septal cholinergic neurons in rats[1,2] and to reduce the occurrence of hippocampal delayed neuronal death following transient ischemia in Mongolian gerbils[3]. It was also reported that basic fibroblast growth factor (bFGF) prevents retrograde degeneration of thalamic neurons after cortical infarction in rats[4]. However, it is very difficult to use such biological polypeptides as NGF and bFGF as therapeutic agents since they must be administered intraventricularly because of the impermeability of these large molecules across the blood-brain barrier and facile degradation of these peptides in the blood flow by peptidases. So the technique of creating microenvironments for tissue growth to produce NGF by plantation of brain cells has recently been reported for the treatment of neurogenerative disease and spinal cord injuries[5]. However, due to ethical problems, it will take long time to apply this technique for human brains.

Extracts of *Ginkgo biloba* leaves are consumed as dietary supplements to counteract chronic, age-related neurological disorders. The *in vivo* neuromodulatory effects of the extracts have recently been elucidated by monitoring at the genome level in mice[6]. Although the active substances could not be identified, several compounds in the extracts must play a vital role in concert. Thus, the traditional herbal medicines draw public attention for the treatment of neurological disorders.

These observations rationalize the idea that low molecular weight compounds exhibiting neurotrophic actions could be developed as promising therapeutic drugs to prevent neuronal cell death occurring after cerebral hemorrhage caused by ischemia, and to ameliorate some types of dementia.

From this point of view, low molecular weight natural products[7~11] have been explored for their ability to mimic NGF by peripheral administration. In our continuous screening program, a novel lanostane NGA0187 was isolated from a fermentation broth of *Acremonium* sp. TF-0356. The structure of NGA0187 was determined by spectroscopic analysis and X-ray diffraction and is shown in Fig. 1.

In this study, we describe the production, structure determination and biological activities of NGA0187.

Experimental Section

General

The IR spectrum was recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. The UV spectrum was obtained with a Hitachi 220A spectrophotometer. MS spectra were determined with a Micromass platform LC mass spectrometer and a Micromass Q-Tof-2 mass spectrometer. NMR spectra were measured on a Jeol JNM-LA500 spectrometer at ambient temperature using the solvent

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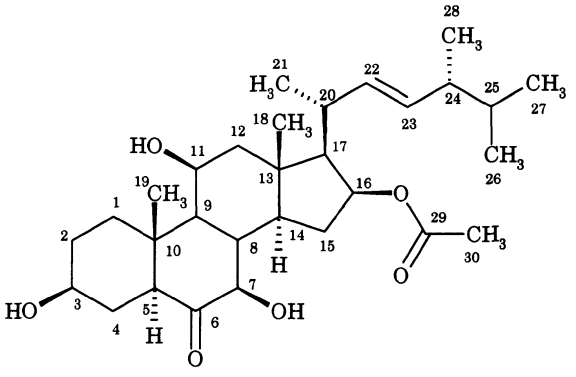
Fig. 1. Structure of NGA0187.



peaks as an internal reference.

X-Ray Crystallography

Crystal Data

The crystal data are summarized as follows: Empirical Formula $C_{30}H_{48}O_6$, FW=504.00, orthorhombic, a= 12.284(2), b=37.949(8), c=6.1958(9) Å, V=2888.3(9) Å^3, Z=4, space group $P2_12_12_1$. The structure was solved by direct methods using the program *maXus*[12] and refined by the full-matrix least-squares method[12]. All H-atom positions were located from a difference Fourier synthesis. All non-hydrogen atoms were refined anisotropically, and H-atoms isotropically. The final R and Rw values were 0.047 and 0.040, respectively.

Taxonomic Studies

The strain TF0356 was originally isolated from a decayed leaf sample collected in Nago-shi, Okinawa Prefecture, Japan. For the evaluation of morphology and cultural characteristics, the strain was grown on malt extract agar, potato glucose agar and LCA (MIURA) agar[13].

Fermentation

A slant culture of the strain TF-0356 was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of seed medium consisting of glucose 2.0%, 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 96 hours on a rotary shaker at 200 rpm. 1.2 liters of the seed culture were transferred into a 200-liter jar fermenter containing 120 liters of the 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.

The culture broth was filtered and the mycelial cake was extracted twice with 36 liters of acetone. Diaion HP-20 (6 liters) was added to the fermentation broth and stirred for 1 hour. The HP-20 resin was collected by filtration and eluted twice with 9 liters of MeOH. The extract of the mycelia and the HP-20 eluate were combined for concentration *in vacuo* to give an aqueous solution that was extracted with ethyl acetate (4 times, 10 liters). The organic layer was evaporated under reduced pressure to yield a residue (76.05 g).

Assay for Neurite Outgrowth

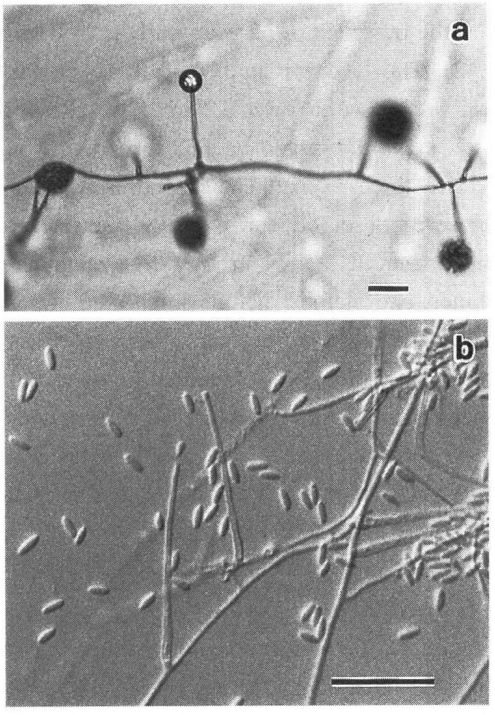
Neurite outgrowth activity was evaluated mainly according to the same methods described by ITO *et al.*[7]. PC12 cells were obtained from the RIKEN Cell Bank and grown in DULBECCO's modified EAGLE's medium (DMEM, GIBCO) with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS), 50 U/ml penicillin and 50 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were plated on collagen-coated 24-well plates (Corning) at a density of 1×10^4 cells/well and cultured for 24 hours. The medium was replaced with a 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 with out 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.

Neuronal Cell Preparation and Cell Survival Assay

The primary culture of cerebral cortical neurons was prepared from 18-day-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, 100 μg/ml streptomycin (DF-FBS medium) were plated on 24-well plates coated with polyethylenimine[14] 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 transferrin, 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[4]. The neurite extension was

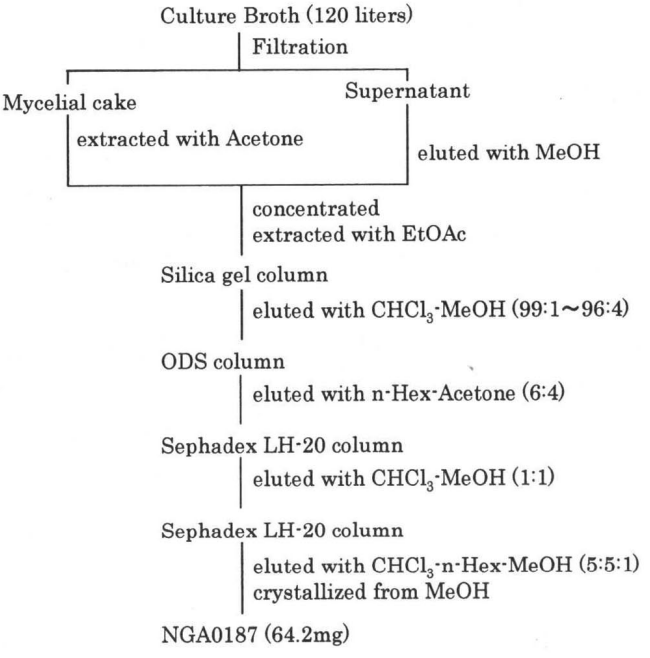
Fig. 2.

(a) Phialides with conidia adhering in slimy heads.
(b) Phialides and conidia.



Bar=50 μm

Fig. 3. Isolation procedure for NGA0187.



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

Results and Discussion

Taxonomy

For the cultural characterization, strain TF0356 was incubated for two weeks at 26°C using three agar media described below.

Colonies on malt extract agar grew slowly, attaining a diameter of 24 mm, and were felty, very pale yellow in color. The reverse side of colonies was the same color as the surface. Colonies on potato-glucose agar grew moderately up to a diameter of 43 mm, and were felty to funiculose, yellowish white to white in color. The reverse side of the colonies was very pale yellow. Colonies on LCA (MIURA) agar grew slowly, reaching a diameter of 30 mm, and were felty, white in color. The reverse side of the colonies was the same color as the surface.

Morphological observations of strain TF0356 grown at 26°C on LCA(MIURA) agar were performed under a light microscope. The conidial ontogeny is enteroblastic. Conidia were produced at the tip of phialides. Phialides are born directly on vegetative hyphae, mostly solitary, hyaline, smooth-walled, 35 to 60 μm long and 2.5 to 3.5 μm wide at the base, and gradually tapering towards the tip to 1.0 to 1.5 μm wide. The minute collarette was showing in the tip of Phiarides. Conidia adhering in slimy heads were single-celled, cylindrical or ellipsoidal, with slightly flattened base, 4.5 to 6.0(−14.0)×1.5 to 2.0 μm. Chlamydospores were not observed. No teleomorph was observed in this strain.

Morphological characteristics and cultural properties of strain TF0356 described above suggested that it belongs to the genus *Acremonium*[15]. The fungus has been deposited at the National Institute of Bioscience and Human-Technology, Japan, as FERM P-13697.

Isolation

All fractionations were guided by bioassay of neurite outgrowth in PC12 cells. The isolation scheme is shown in Fig. 3. The crude solid was purified on a silica gel column packed with $CHCl_3$ and eluted with $CHCl_3$-MeOH by

stepwise increase of MeOH concentrations from 0~10%. The active fractions eluted with 1% to 4% MeOH in $CHCl_3$ were concentrated to yield 25 g of brown powder, which was then dissolved in *n*-hexane - acetone (6 : 4) and applied to a preparative HPLC column (glass column ODS-A60, 50 i.d.×500 mm). The column was eluted with *n*-hexane - acetone (6 : 4) and the active fraction was concentrated to afford a semi-purified powder. The powder was applied to a Sephadex LH-20 column and developed with $CHCl_3$ - MeOH (1 : 1). The active fractions were combined and concentrated. This material was then re-chromatographed over a Sephadex LH-20 with $CHCl_3$ - MeOH - *n*-hexane (5 : 1 : 5). The pure NGA0187 was crystallized from MeOH as colorless needles (64.2 mg).

Table 1. Physico-chemical properties of NGA0187.

Appearance		colorless needles
Molecular formula		$C_{30}H_{48}O_6$
HR ESI-MS	Observed	527.3358 $(M+Na)^+$
	Calcd	527.3349
UV λ_{max}(MeOH)nm(ε)		206 (1700)
		207 (100)
IR ν_{max}(KBr)cm^{-1}		3485, 2966, 2872, 1714, 1698, 1459, 1376, 1269, 1168, 1116, 1074, 1023

Structure Determination

The physico-chemical properties of NGA0187 are summarized in Table 1. The molecular formula was determined as $C_{30}H_{48}O_6$ on the basis of its high resolution ESI mass and NMR data. The IR and ^{13}C-NMR spectra suggested the presence of an ester carbonyl (ν 1714 cm^{-1}, δ_C 169.42). The ^{1}H-NMR and ^{13}C-NMR spectral data of NGA0187 are shown in Table 2. The ^{1}H-NMR spectrum of NGA0187 exhibited seven methyl groups, two olefinic protons, and three exchangeable protons. All 30 carbon signals were observed on the ^{13}C-NMR spectrum, consisting of seven methyl groups, five methylenes, four oxymethines, two olefinic methines, ten methines, and four quaternary carbons including two carbonyl carbons. As the degree of unsaturations was estimated to be seven from its molecular formula, two degrees were assigned to two carbonyl groups and one to one double bond, leaving the residual four to accommodate four rings. The structure of NGA0187 was elucidated by extensive NMR analysis. Three partial structures were deduced by tracing each of correlation networks in the ^{1}H-^{1}H COSY spectrum as shown in Fig. 4. The long-range correlation maps from two singlet methyl lines at H_3-18 and H_3-19 were observed in the HMBC spectrum as shown in Fig. 4. A carbonyl group at C-6 showed long-range spin networks from H-5 and 7-OH, respectively. Although ^{1}H signals for 3-OH and 7-OH were overlapped, it was possible to discriminate between their HMBC correlations and trace their connections by analysis of correlation peaks in the HMQC and COSY spectra. The position of an acetyl group was determined by

Table 2. ^{13}C- and ^{1}H-NMR data for NGA0187 (in DMSO-d_6).

Carbon No.	δC (100MHz)	δH (J in Hz, 500MHz)	Carbon No.	δC (100MHz)	δH (J in Hz, 500MHz)
1	35.21 (t)	1.82 (1H,m)	16	74.61 (d)	4.86 (1H,m)
		1.22 (1H,m)	17	58.96 (d)	1.19 (1H,dd; 7.9, 11.0)
2	30.32 (t)	1.62 (1H,m)	18	14.91 (q)	1.03 (3H,s)
		1.22 (1H,m)	19	15.19 (q)	0.79 (3H,s)
3	68.50 (d)	3.29 (1H,m)	20	33.98 (d)	2.43 (1H,m)
4	29.61 (t)	1.62 (1H,m)	21	20.74 (q)	1.02 (3H,d; 7.3)
		1.22 (1H,m)	22	135.75 (d)	5.15 (1H,m)
5	53.72 (d)	2.29 (1H,dd; 2.5, 12.0)	23	131.75 (d)	5.15 (1H,m)
6	210.35 (s)		24	42.56 (d)	1.74 (1H,m)
7	78.36 (d)	3.67 (1Hdd; 5.0, 8.6)	25	32.40 (d)	1.39 (1H,m)
8	41.68 (d)	1.91 (1H,m)	26	19.91 (q)	0.78 (3H,d; 6.7)
9	54.59 (d)	1.30 (1H,m)	27	19.45 (q)	0.76 (3H,d; 6.1)
10	39.54 (s)		28	17.70 (q)	0.81 (3H,d; 6.7)
11	66.33 (d)	4.16 (1H,m)	29	169.42 (s)	
12	47.67 (t)	2.12 (2H, brd; 11.6)	30	21.19 (q)	1.92 (3H,s)
13	42.68 (s)				
14	55.12 (d)	1.30 (1H,m)	3, 7-OH		4.53 (2H,d; 5.0)
15	36.91 (t)	2.39 (1H,m)	11-OH		4.24 (1H,d; 2.4)
		1.29 (1H,m)			

Fig. 4. Structure elucidation of NGA0187 by NMR analyses.

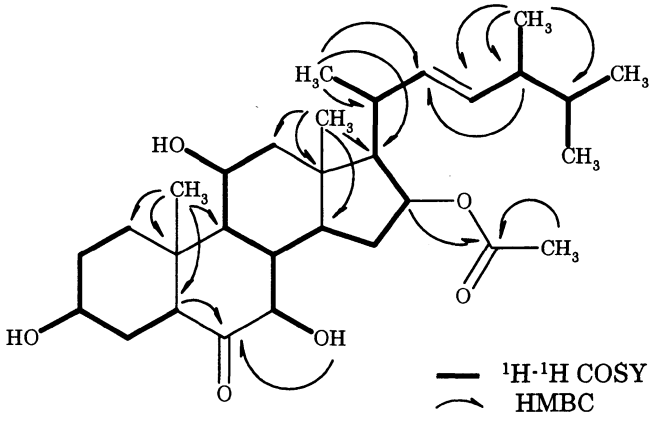
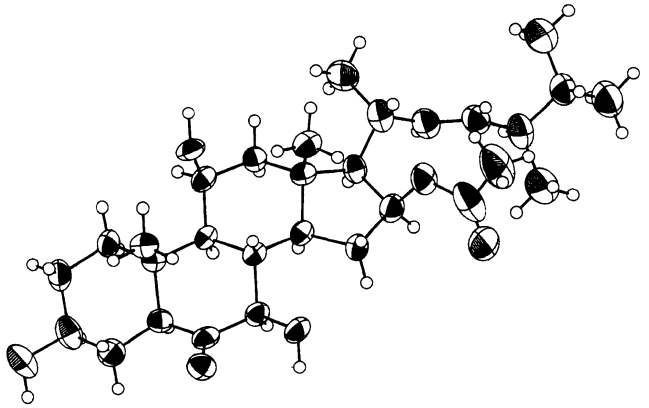
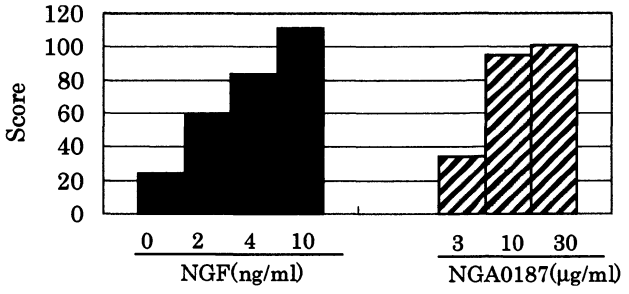


Fig. 5. X-Ray crystal structure of NGA0187.



tracing the correlation maps from H-16 to a carbonyl carbon at δ_C 169.42. The location of an alkyl side chain was deduced by analysis of the spin networks from H-21 to C-17, and from H-17 to C-20 and C-22. The extensive analysis of these NMR data led to the proposal of a lanostane skeleton and finally to the elaboration of the planar structure of NGA0187. As NGA0187 furnished suitable crystals, its relative stereochemistry was determined by single-crystal X-ray analysis as shown in Fig. 5. The ORTEP diagram revealed the relative stereochemistry of three hydroxyl groups to be 3β, 7β and 11β. In addition, the junctions of the A/B and the C/D rings were determined to be trans-fused, having a flat conformation of the skeletal system. NGA0187 is a C30 lanostane triterpene. Polyhydroxysteroids that have a

Fig. 6. Effect of NGA0187 neurite outgrowth in PC12 cells.



similar structure were found in starfish[16]. The structure of NGA0187 is unique as a fungal metabolite and in that it contains hydroxyl groups at C-7 and C-11, a carbonyl group at C-6, and an acetyl group at C-16. During our submission of this paper, the same compound anicequel[17], isolated from a culture broth of *Penicillium aurantiogriseum* Dierckx TP-F0213, was reported as an inhibitor for anchorage-independent growth of tumor cell.

Biological Properties

NGA0187 generated neurite outgrowth of PC12 as shown in Fig. 6. NGA0187 induced a significant neurite outgrowth at a dose of 30 μg/ml, corresponding to NGF at 10 ng/ml. Although cell survival activity of NGA0187 was examined using rat cerebral cortical neuronal cells, NGA0187 did not show protective effects against neuronal cell damages (10 μg/ml, data not shown). Recently, a tetracyclic triterpene S19159 was isolated as a modulator of neurite outgrowth from the fermentation broth of a fungal strain[18]. It was reported that S19159 exhibited the rapid and extensive morphological and cytotoxic effects on cerebral cortical neurons, but did not have any effect on PC12 cells. It is suggested that these biological effects of S19159 on neurons were caused by inhibition of cholesterol biosynthesis. The mode of action of NGA0187 on neurite outgrowth is under investigation.

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