NG-011 AND NG-012, NOVEL POTENTIATORS OF NERVE GROWTH FACTOR

I. TAXONOMY, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

MAYUMI ITO, MASAFUMI MARUHASHI, NORIYOSHI SAKAI, KAZUTOSHI MIZOUE* and KAZUNORI HANADA

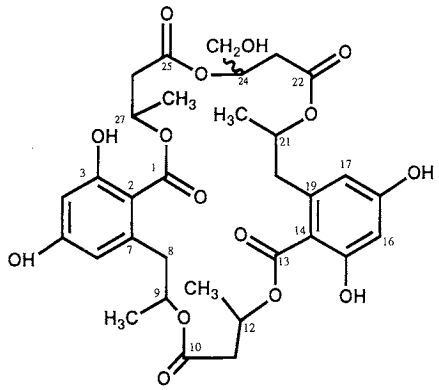
Research Center, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Ohmiya-shi, Saitama 330, Japan

(Received for publication March 25, 1992)

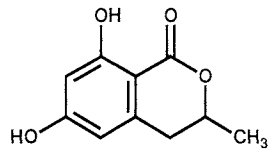
NG-011 and NG-012, novel potentiators of nerve growth factor (NGF), were isolated from the culture broth of *Penicillium verruculosum* F-4542, together with 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin. They potentiated the neurite outgrowth induced by NGF in rat pheochromocytoma cell line (PC12).

Nerve growth factor (NGF) is known to be a prototypical neurotrophic factor essential for growth and development of neurons in the central as well as peripheral nervous system[1]. Furthermore, NGF treatment was found to ameliorate the age-related impairment in memory tests and to prevent the lesion-induced loss of septal cholinergic neurons in rats[2~4]. The etiology and pathogenesis of ALZHEIMER'S disease are poorly understood, but symptomatic disease is associated histopathologically with neuronal loss in the temporal lobe and neocortex of the brain[5]. So NGF may prevent neuronal loss if it is given to ALZHEIMER patients. However, it is difficult to clinically use NGF as a drug because of intraventricular administration. From this point of view, we started to explore low molecular compounds which can mimic or potentiate NGF by peripheral administration. In our screening program for these compounds in the microbial metabolites, a culture broth of *Penicillium verruculosum* F-4542 was found to enhance the neurite outgrowth in the presence of NGF in rat pheochromocytoma cell line PC12. Bioassay-guided fractionations of the ethyl acetate extract of the culture broth led to the isolation of novel potentiators, NG-011 and NG-012, together with 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin as shown in Fig. 1. In this paper

Fig. 1. Structures of NG-011, NG-012 and 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin.



NG-011 and NG-012
NG-011 is an epimer of NG-012 at C24



3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin

we describe the taxonomy of producing organism, isolation, and physico-chemical and biological properties of NG-011 and NG-012.

Taxonomy of the Producing Organism

Strain F-4542 was isolated from a soil sample collected at Yamaguchi-shi, Yamaguchi Prefecture, Japan. For the identification of the fungus, CZAPEK yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) were used[6].

Colonies on CYA medium grow rapidly, attaining a diameter of 38~42 mm in 7 days at 25°C and are velutinous to floccose, grayish green to reddish green in color, nearly white at the margin. Reverse of colonies are pale reddish yellow. A reddish exudate is present mostly over the central area.

Colonies on MEA medium are velutinous to lightly funiculose, 45~47 mm in diameter, and yellowish green in color, nearly yellow at the margin due to bright yellow mycelium. Reverse of colonies are pale reddish yellow. Exudate is not present.

Colonies on G25N are 4~6 mm in diameter, white to blueish green in color and pale yellowish white on reverse.

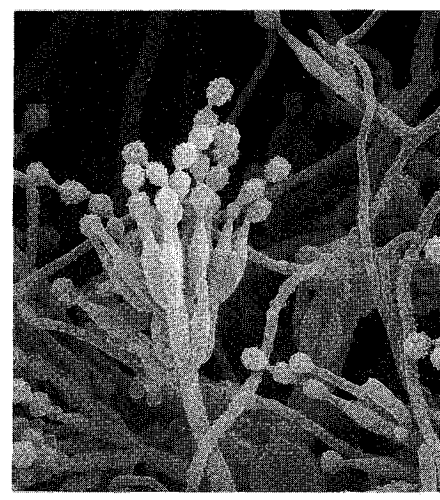
At 37°C on CYA medium, colonies are 42~45 mm in diameter. Their characteristics are similar to those on CYA medium at 25°C except for more floccose, and exudate is not present. They are not germed at 5°C.

Conidiophores are 90~205 μm in length, 2.0~3.5 μm in width and slightly rough-walled. Penicilli are symmetrically biverticillate. Metulae are 2~10 in divergent verticils and 8.0~10.0 μm × 3.0~4.0 μm. Phialides are 1~10 per metula, acerose and 7.0~13.0 μm × 3.0~4.5 μm. Conidia are spherical to subspherical and 2.8~3.5 μm in diameter with walls roughened or spinose and born in disordered chains.

From the above-mentioned characteristics, strain F-4542 belongs to the genus *Penicillium*. Furthermore, based on the features of penicilli and conidia, and the color of the mycelia on MEA medium, the strain was identified as *Penicillium verruculosum* F-4542. The scanning electron micrograph of *Penicillium verruculosum* F-4542 is shown in Fig. 2. This strain was also deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM P-11273.

Fig. 2. Scanning electron micrograph of *Penicillium verruculosum* F-4542.

Bar represents 15.0 μm.



Fermentation

A loopful of a slant culture of *Penicillium verruculosum* F-4542 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a 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 flask was shaken on a rotary shaker at 28°C for 72 hours. One liter of the seed culture was transferred into a 200-liter tank fermenter containing 120 liters of a production medium with the same composition as in the seed medium.

Fermentation was carried out at 28°C for 72 hours under aeration of 1.0 v/v/minute and agitation of 500 rpm.

Isolation

All fractionations were guided by potentiation of NGF-mediated neurite outgrowth in PC12 cells.

The isolation procedure of NG-011 and NG-012 is shown in Fig. 3.

The culture broth was centrifuged to separate mycelial cake and supernatant. The mycelial cake was extracted with 70% aqueous acetone (5 liters), followed by filtration and concentration to an aqueous solution. The solution was combined with the supernatant (80 liters, pH 5.0) and mixed with 4.5 liters of Diaion HP-20. The resin was washed with water and eluted with methanol (9 liters). The eluate was concentrated *in vacuo* to an aqueous solution and extracted with ethyl acetate (4 liters, 3 times). The organic layer was evaporated under reduced pressure to yield a residue (18.0 g). This residue was dissolved in 20 ml of *n*-hexane - methylene chloride - ethanol (3 : 8 : 0.5) and applied to a column of silica gel (5 × 40 cm) packed with the same solvent. The column chromatography was developed with the same solvent. The active fractions were concentrated and dissolved in 6 ml of *n*-hexane - methylene chloride - ethanol (3 : 8 : 1), which was then chromatographed on Sephadex LH-20 (4 × 40 cm) by the same solvent. The eluate was monitored by color reaction with anisaldehyde-sulfate on TLC plate as well as bioassay. The first anisaldehyde-sulfate-positive fractions were collected and concentrated to give an active compound (29 mg), which was identified with 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin[7,8] by its physico-chemical properties. The second anisaldehyde-sulfate-positive fractions were collected and evaporated *in vacuo*. The further purification was performed on a ODS column chromatography (Fuji-Davison, Chromatorex, 100 ~ 200 mesh, 2.5 × 25 cm) using 50% acetonitrile as an eluant to give a white powder of NG-011 (980 mg). The last anisaldehyde-sulfate-positive fractions were collected and concentrated to give a white powder of NG-012 (3.2 g).

Fig. 3. The isolation procedure of NG-011 and NG-012.

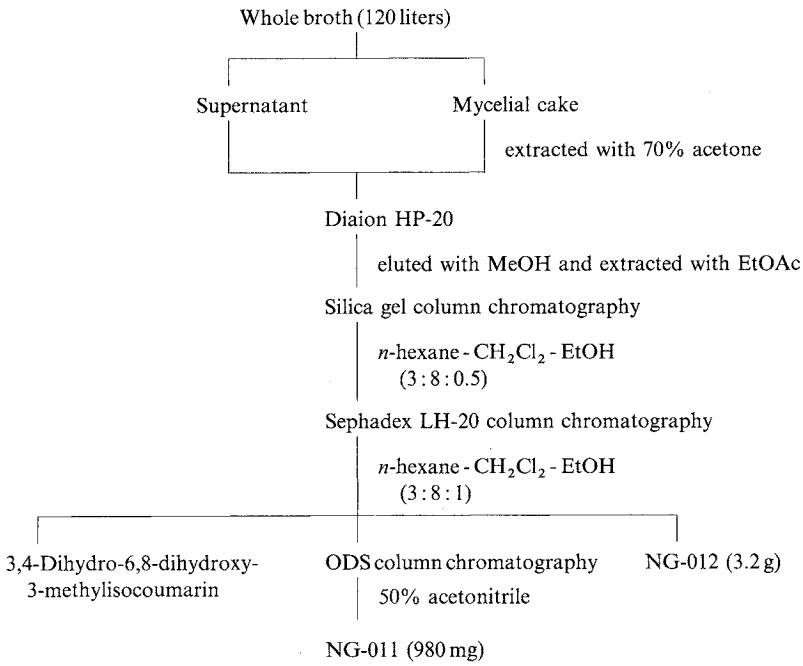


Table 1. Physico-chemical properties of NG-011 and NG-012.

	NG-011	NG-012
Appearance	White powder	White powder
MP	85~100°C	113~120°C
$[\alpha]_D^{26}$	−7.2° (*c* 0.25, EtOH)	−25.2° (*c* 0.25, EtOH)
Molecular formula	$C_{32}H_{38}O_{15}$	$C_{32}H_{38}O_{15}$
HREI-MS (*m/z*)		
Found:	662.2245	662.2224
Calcd:	662.2211 for $C_{32}H_{38}O_{15}$	662.2211 for $C_{32}H_{38}O_{15}$
FAB-MS (*m/z*)	663 $(M+H)^+$	663 $(M+H)^+$
UV λ_{max}^{MeOH} nm (ε)	217 (41,900), 264 (22,400), 302 (10,000)	217 (36,700), 264 (19,300), 302 (8,600)
IR ν_{max}^{KBr} cm^{-1}	3413, 2984, 1729, 1651, 1621	3411, 2984, 1734, 1651, 1621
TLC (Rf)	0.46[a]	0.34[a]

[a] Merck, kieselgel 60 F_{254}: *n*-Hexane - CH_2Cl_2 - EtOH (3:8:1).

Physico-chemical Properties

The physico-chemical properties of NG-011 and NG-012 are summarized in Table 1. They are white powders which are readily soluble in methanol, ethanol, ethyl acetate, acetone and diethyl ether, slightly soluble in chloroform, benzene and *n*-hexane and insoluble in water. They give positive color reactions to iodine, sulfuric acid, anisaldehyde-sulfate, $FeCl_3$ but negative to ninhydrin.

The molecular formula of both NG-011 and NG-012 were determined to be $C_{32}H_{38}O_{15}$ on the basis of their HREI-MS spectra and elemental analysis. The UV spectra of them were observed with three maximum peaks at 217, 264 and 302 nm in methanol. These spectra were similar to that of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin, suggesting that NG-011 and NG-012 contain the same chromophore as 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin. The IR spectra of NG-011 and NG-012 showed characteristic absorptions attributed to hydroxyl groups (3413 cm^{-1} and 3411 cm^{-1}) and ester carbonyl groups (1729 cm^{-1} and 1734 cm^{-1}). Both of the ^1H NMR and ^{13}C NMR spectra of NG-011 and NG-012 showed very similar spectral patterns except for some regions as depicted in Figs. 4 and 5. Furthermore their EI and FAB-MS spectra showed the same fragmentation patterns. These spectral characteristics suggested that their structural relationship is epimeric.

The structures of NG-011 and NG-012 were elucidated by their spectral analysis as shown in Fig. 1. Their structural determination will be described in the succeeding paper[9].

Biological Properties

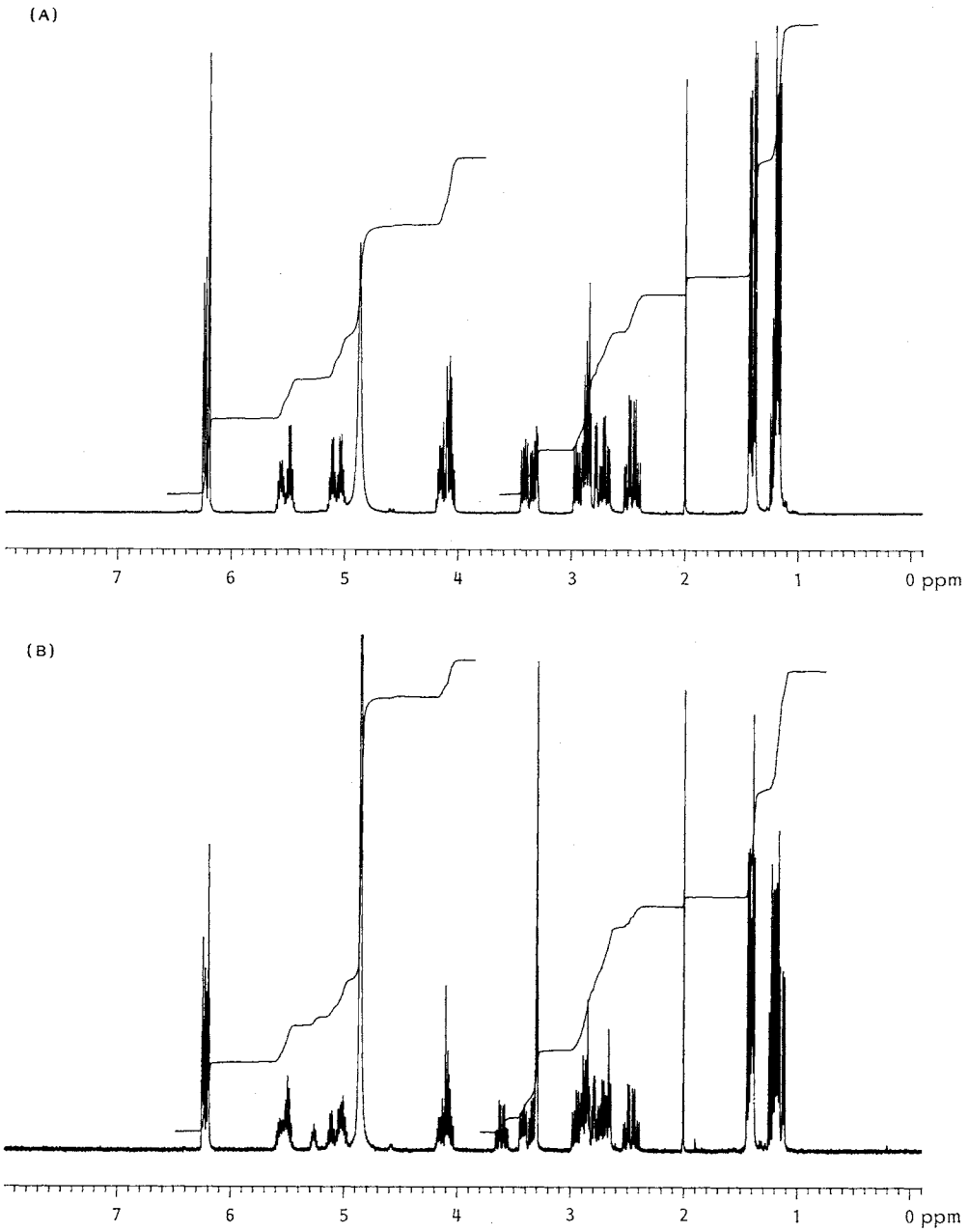
As depicted in Table 2, NG-011, NG-012 and 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin were found to potentiate NGF in PC12 cells. These effects were dose-dependent with a concentration of 100 μg/ml (NG-011 and NG-012) or 300 μg/ml (3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin) giving a peak response. The maximal effects were equal in magnitude to that of NGF at 2 ng/ml (NG-011 and NG-012) or 10 ng/ml (3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin). Higher concentration of NG-011 or NG-012 (300 μg/ml) caused cell death (data not shown).

NG-011 and NG-012 had no antimicrobial activities against selected bacteria, fungi and yeasts tested by the conventional paper disc method at a concentration of 1 mg/ml (data not shown).

Discussion

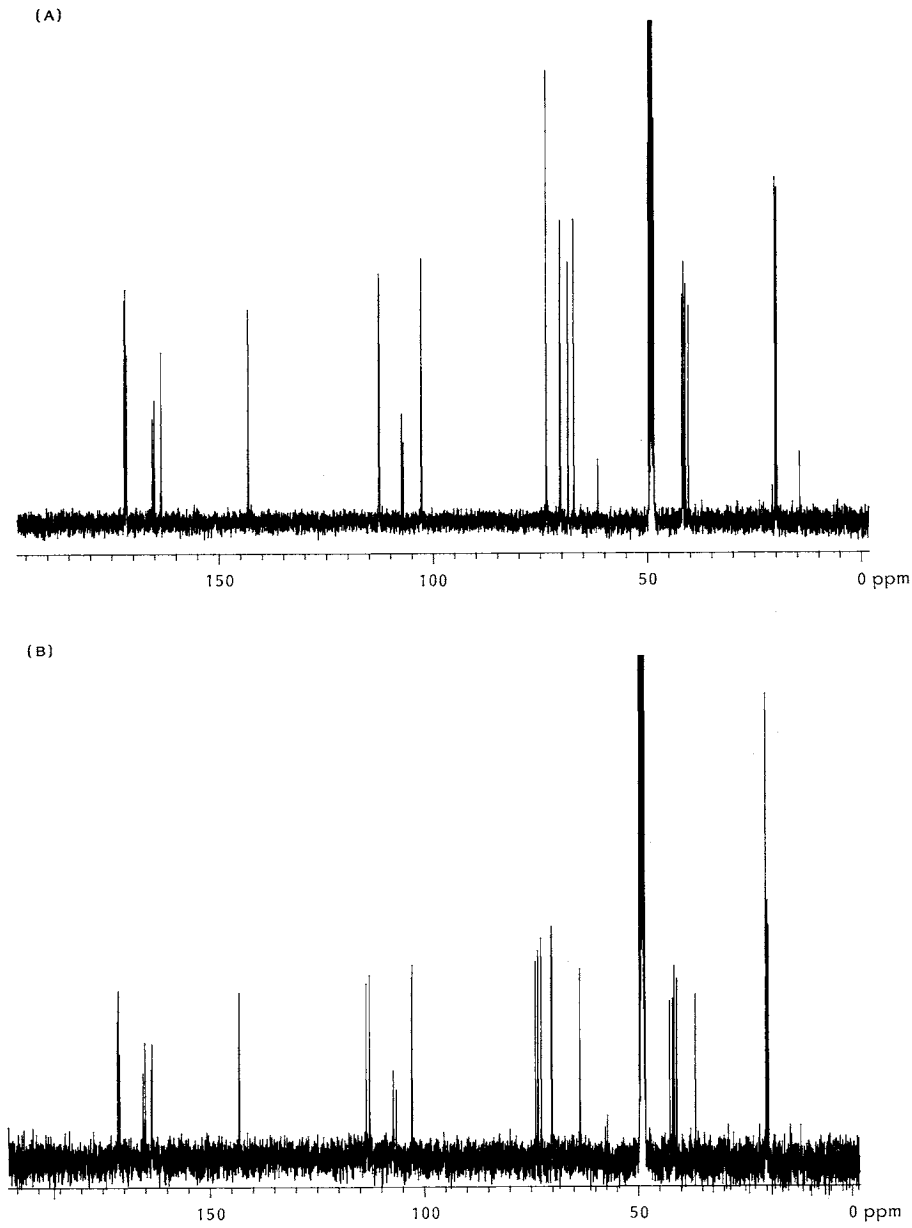
In the course of our exploration for compounds enhancing effect of NGF on the outgrowth of

Fig. 4. ^1H NMR spectra of (A) NG-011 and (B) NG-012 (CD_3OD).



neurite in PC12 cells, novel potentiators of NGF, NG-011 and NG-012, together with 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin, were isolated from the culture broth of *Penicillium verruculosum* F-4542. NG-011 and NG-012 are epimers to each other which form a unique macrolactone containing 2 molecules of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin in their structure. Recently, 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin has been reported to be a major metabolite of *Ceratocystis* which causes plant disease of pine trees in Western Canada[8], but its pharmacological properties have not been described. 3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin was found to potentiate NGF as well as NG-011 and NG-012, indicating that a 2,4-dihydroxy-6-(2-hydroxy-*n*-propyl)benzoic moiety is essential for their activities. When they are applied to PC12, NG-011 and NG-012 might be incorporated into the

Fig. 5. ^{13}C NMR spectra of (A) NG-011 and (B) NG-012 (CD_3OD).



cell and hydrolyzed by their esterases to give 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin and exhibit their biological activities. Their mode of action is now under study, which will be presented elsewhere.

Experimental

General

MP's were determined with a Yanagimoto micro-melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectra were recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. UV spectra were measured on a Hitachi 220A spectrophotometer. EI-MS, FAB-MS and HRFAB-MS spectra were determined with a Jeol JMS-SX 102

Table 2. Effect of NG-011, NG-012 and 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin on NGF-mediated neurite outgrowth in PC12 cells.

Test compound	Dose (μg/ml)	Score
Control (0.5 ng/ml NGF)		37± 1
NG-011	10	28± 3
	30	46±13
	100	71± 9**
NG-012	10	30± 7
	30	52± 9*
	100	63± 4***
3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin	10	33±13
	30	41±16
	100	76±12**
	300	107±11***

All compounds as well as control were tested in the presence of 0.5 ng/ml NGF. Significantly different from control ($n=3$, Mean±SD): * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

mass spectrometer. NMR spectra were obtained with a Jeol JNM-GX400 with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz using the solvent peaks as internal references downfield of TMS at 0 ppm.

Assay for Potentiation of NGF-mediated Neurite Outgrowth in PC12 Cells

PC12 cells were obtained from RIKEN Cell Bank and maintained as monolayer culture in DULBECCO's modified EAGLE's medium (DMEM, GIBCO) with 10% fetal bovine serum (FBS), 5% horse serum (HS) and antibiotics. The cells were kept in a humidified incubator at 37°C and 5% CO_2. To assess potentiation of NGF, the cells were reseeded on collagen-coated 24-well plates (Corning) at a density of 1×10^4 per well. The seeding medium was changed 24 hours after reseeding to the test medium, which consisted of DMEM with 10% FBS, 5% HS, antibiotics, a subthreshold dose of NGF (0.5 ng/ml) and a methanolic solution of the sample (0.01 ml/ml medium). After 48 hours in culture, 100 cells were counted with 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).

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