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Indocarbazostatin and Indocarbazostatin B, Novel Inhibitors of NGF-induced Neuronal Differentiation in PC12 Cells

I. Screening, Taxonomy, Fermentation and Biological Activities

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During the course of our screening for modulators of signal transduction of mammalian cells, we discovered two novel indolocarbazole antibiotics, indocarbazostatin and indocabazostatin B, from a culture broth of a *Streptomyces* sp. as inhibitors of NGF-induced neuronal differentiation in rat pheochromocytoma PC12 cells. Indocarbazostatin and indocarbazostatin B inhibited NGF-induced neurite outgrowth from PC12 cells at 6 nM and 24 nM, respectively, whereas K-252a inhibited at 200 nM under our assay conditions.

Neurotrophic factors such as the nerve growth factor (NGF) are known to be essential for the survival and differentiation of sympathetic and sensory neurons. Decrease in availability of neurotrophic factors is considered to cause dysfunction of the nervous system, resulting in various nerve diseases including senile dementia such as Alzheimer's[1] and Parkinson's[2] diseases. On the other hand, up-regulation of neurotorophic factors was observed in patients with intractable temporal lobe epilepsy[3] and after excitotoxicity in a rat model of Huntington's disease.[4] This observation led us to speculate that a modulator for neurite outgrowth can be useful to treat patients with neuropathy. Although several modulators, including KS-505a,[5] K-252a,[6,7] staurosporine,[8] lactacystin,[9] epolactaene,[10] PD-098059,[11] PD-90780[12] and AG879[13] have been discovered so far, there are delays in practical application as therapeutic drugs. Under these circumstances, we performed screening for natural substances that regulate differentiation of rat pheochromocytoma PC12 cells. During the course of screening of 4671 extracts (2532 extracts from actinomycetes, 1958 extracts from fungi and 181 extracts from plants), only one sample showed activity of interest. The producing strain identified as *Streptomyces* sp. TA-0403 was cultivated at 30°C for 3 days in 500-ml K-1 flasks (incubator bottles) each containing 70 ml of the producing medium. The fermentation broth (35 liters) was centrifuged and the mycelial cake was extracted with acetone. After separation and purification of the active principles from the acetone extracts, indocarbazostatin[14] (**1**) and indocarbazostatin B (**2**) were isolated (Fig. 1). Details of the isolation, physico-chemical properties and structure elucidation are reported in the following paper.[15] In the present paper we describe details of the taxonomy and the fermentation conditions of the producing organism, and the biological activities of these compounds.

Material and Methods

Chemicals

Indolocarbazole antibiotics, K-252a, K-252b and staurosporine, were purchased from CosmoBio Inc.

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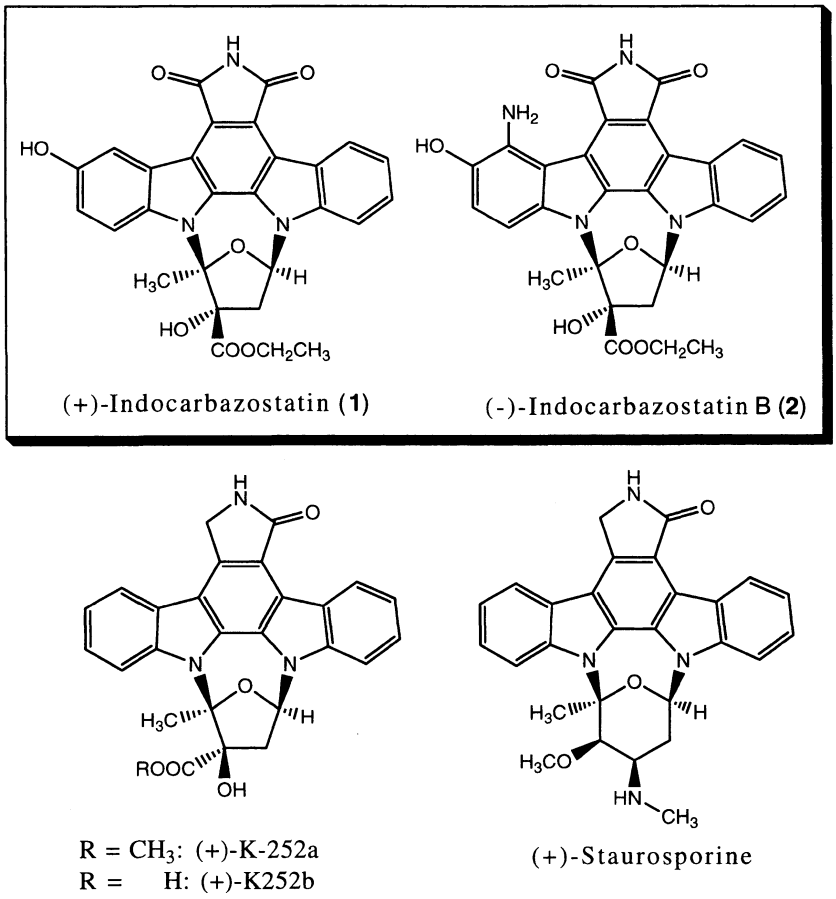
Fig. 1. Structures of indocarbazostatin (**1**) and indocarbazostatin B (**2**), and standard substances, K252a, K252b and staurosporine.



Taxonomic Studies

The producing strain, TA-0403, was isolated from a soil sample collected at Aki-gun, Kochi Prefecture, Japan. Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB.[16] Carbohydrate utilization was investigated as described by PRIDHAM and GOTTLIEB.[17] Morphological characteristics were observed with a scanning electron microscope (Model S-2500, Hitachi). The isomer of diaminopimeric acid was analyzed by the method of STANECK and ROBERTS.[18] Menaquinones were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.*[19]

Fermentation Procedures of the Producing Strain

A slant culture of strain TA-0403 was inoculated into a 500-ml K-1 flask (purchased from K-Techno Corporation) containing 70 ml of culture medium consisting of glucose 0.5%, soluble starch 2%, NZ Case 0.3%, yeast extract 0.3%, fish meal 0.5%, $CaCO_3$ 0.2% (pH 6.5 before sterilization). The flask was shaken on a rotary shaker (200 rpm) at 30°C for 2 days. This seed culture (2 ml) was transferred into 500-ml K-1 flasks each containing 70 ml of production medium consisting of glucose 0.29%, soluble starch 1.44%, Pharmamedia 1.58%, soybean meal 0.79%, corn steep liquor 0.79%, yeast extract 0.32%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3% (pH 7.0). Fermentation was carried out at 30°C for 3 days on a rotary shaker.

Inhibitory Activities of NGF-induced Neurite Outgrowth from PC12 Cells

PC12 cells (purchased from RIKEN Cell Bank, Japan) were grown in DMEM (Dulbeco's Modified Eagle Medium) supplemented with 0.35% D-glucose, 10% FBS (Fetal Bovine Serum) and 10% HS (Horse Serum) at 37°C in a 5% CO_2 atmosphere. The cells (200 μl of 0.5×10^4 cells/ml) were plated into each well of a 96-well collagen

Type I coated plate (IWAKI). After 12 hours, 2 μl of sample dissolved in 10% DMSO was added. At 12 hours after sample addition, 10 μl of NGF (Upstate Biotechnology Incorporated, USA) was added in each well, and effects of the samples on NGF-induced neurite outgrowth were observed under a microscope.

Cytotoxicity against PC12 Cells

Cytotoxicity of indocarbazostatin was measured by MTT[20] and Calcein/AM methods.[21] The cells (200 μl of 1×10^5 cells/ml) were plated on a 96-well plate. After 12 hours, samples were added in each well. Twenty-four hours after addition of 2 μl of samples dissolved in 10% DMSO, the cells were washed twice with PBS (−), and viability of the cells was measured by MTT and Calcein/AM methods. MTT method: To the cells was added 90 μl of the medium and 10 μl of MTT solution (5 mg/ml of PBS (−)), and the plate was incubated at 37°C for 4 hours in a CO_2 incubator. Isopropanol containing 0.04 N HCl was added to each well. Absorption at 560 nm was measured with a microplate reader. Calcein/AM method: To the cells was added 90 μl of PBS (−) and 10 μl of the Calcein/AM solution (25 μM in DMSO) and the plate was incubated at 37°C for 1 hour in a CO_2 incubator. Fluorescence at 530 nm was measured with excitation at 485 nm with a fluorescence multi-well plate reader (CytoFluor II, Applied Biosystems).

Growth Inhibition on PC12 Cells

The cells (each 200 μl of 3×10^4 cells/ml) were plated on a 96-well plate (PACKARD), 2 μl of [^{3}H]thymidine (5 μCi/ml) was added and the plate was incubated at 37°C for 2 hours in a CO_2 incubator. After removal of the solution, the cells were washed twice with 200 μl aliquots of PBS (−), and then added 200 μl of the medium. The incorporation of [^{3}H] thymidine was repeated 5 times every 24 hours. After 5 days, the cells were washed twice with PBS (−), and to each well was added 100 μl of MICROSCINT^{TM}-20 (PACKARD). The incorporation of [^{3}H]thymidine was measured on a micro plate scintillation counter (TOPCOUNT, PACKARD). Sample was added at 48 hours.

Inhibition of Protein Kinase C

Protein kinase C from rat brain was purchased from Molecular Probe Inc., U.S.A. [γ-^{32}P] ATP was obtained from Amersham Pharmacia Biotech UK. Ltd. Inhibitory activity of protein kinase C was assayed essentially as described by the PKC assay kit protocol (Upstate Biotechnology, U.S.A.). Briefly, the incubation mixture of 10 μl of substrate cocktail, 10 μl of inhibitor cocktail, and 10 μl of enzyme preparation with or without a sample of 1 μl in DMSO was incubated in the bottom of a microcentrifuge tube on ice. The reaction was started with or without the sample by adding 10 μl of the Mg^{2+}/ATP cocktail containing [γ-^{32}P] ATP, the microcentrifuge tube was vortexed gently and incubated at 30°C for 10 minutes. The reaction was stopped by blotting a 25 μl aliquot on P81 paper square for 30 seconds before immersing the paper into a beaker containing 0.75% phosphoric acid (×10). After washing, acetone was added and washed for 2 minutes (×2). Scintillation cocktail was added and the bound radioactivity on the paper was quantitated in a scintillation counter for 1 minute.

Results

Screening

We screened for natural substances that regulate differentiation of rat pheochromocytoma PC12 cells. Among 4671 extracts, only those produced by a single microbial strain, TA-0403, contained active compounds of interest. PC12 cells (purchased from RIKEN Cell Bank) were seeded at 0.5×10^4 cells/ml in Dulbecco's modified Eagle's medium supplemented with 0.35% D-glucose, 10% FBS and 10% HS at 37°C in a 96-well collagen-coated plate, and treated with test samples after 24 hours. Production of inhibitors and optimization of the producing medium were monitored by the bioassay.

Taxonomic Studies of the Producing Strain

The cultural characteristics of strain TA-0403 grown on various media at 28°C for 14 days are shown in Table 1. The strain formed well-branched substrate mycelia. The aerial mycelia grew abundantly on inorganic salt-starch agar (ISP No. 4). The spore chains were spiral with 20~40 or more spores per chain. The spores were subspherical to oval in shape, 0.8~1.2 μm in size. The spore surface was spiny. No fragmentation of substrate mycelia, sporangia, sclerotia or motile spores was observed (Fig. 2).

The physiological and carbohydrate utilization characteristics are shown in Table 2. Analysis of the whole-cell hydrolysate of the strain showed the presence of LL-diaminopimelic acid. The predominant menaquinone was MK-9 (H_8) and a small amount of MK-9 (H_6) was also present. Based on these characteristics, strain TA-0403 belongs to the genus *Streptomyces*. This strain has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and

Table 1. Cultural characteristics of strain TA-0403.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar (ISP No. 2)	Poor	Poor, gray	Light yellow	None
Oatmeal agar (ISP No. 3)	Poor	Poor, gray	None	Yellowish green
Inorganic salts-starch agar (ISP No. 4)	Moderate	Good, gray	Pale yellow	None
Glycerol-asparagine agar (ISP No. 5)	Poor	Moderate, gray	Pale yellow	None
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	None	None	None
Tyrosine agar (ISP No. 7)	Moderate	Poor, light gray	Yellowish white	None
Sucrose- nitrate agar	Moderate	None	Yellowish white	None
Glucose-asparagine agar	Poor	Moderate, gray	None	Light yellow
Nutrient agar	Poor	None	None	None

Technology, Japan, under the name of *Streptomyces* sp. TA-0403 with the accession No. FERM P-17190.

Fermentation

A slant culture of strain TA-0403 was inoculated into a 500-ml K-1 flask containing 70 ml of culture medium consisting of glucose 0.5%, soluble starch 2%, NZ Case 0.3%, yeast extract 0.3%, fish meal 0.5%, $CaCO_3$ 0.2% (pH 6.5 before sterilization). The flask was incubated at 30°C for 2 days on a rotary shaker (200 rpm). This seed culture (2 ml) was transferred into 500 of 500-ml K-1 flasks each containing 70 ml of production medium consisting of glucose 0.29%, soluble starch 1.44%, pharmamedia 1.58%, soybean meal 0.79%, corn steep liquor 0.79%, yeast extract 0.32%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3% (pH 7.0). Fermentation was carried out at 30°C for 3 days on a rotary shaker.

Biological Activities

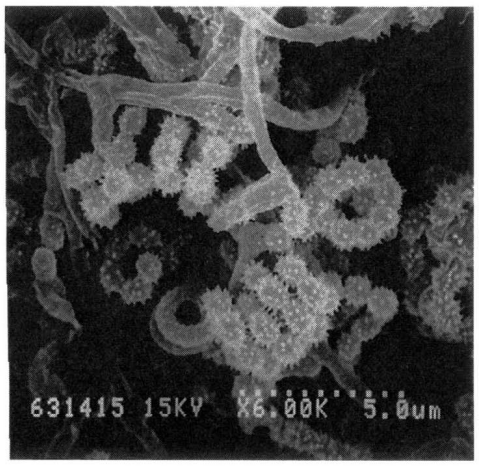
The two new compounds, indocarbazostatin (**1**) and indocarbazostatin B (**2**) were isolated as described in the following paper.[15] The compounds **1** and **2** inhibited NGF-induced neurite outgrowth from PC12 cells at 6 nM and 24 nM, respectively, whereas K-252a inhibited at 194 nM under our assay conditions (Table 3). K-252b did not show any observed activity at 194 nM but exhibited cytotoxicity at 388 nM under the microscopic observation.

Cytotoxicities of **1** and K-252a were measured by the MTT method[20] and Calcein/AM method.[21] Neither inhibitor showed any trace of cytotoxicity at their minimal

effective concentration. However, K-252a showed complete cytotoxicity at the concentration three times higher than the minimal effective concentration, whereas **1** showed only approximately 60% of cells had injury at the concentration ten times higher than the minimal effective concentration (Table 4).

K-252a showed cytotoxicity, which was evaluated with incorporation of [^{3}H] thymidine into PC12 cells, at a concentration three times higher than the minimal effective concentration as shown in Fig. 3B. However, **1** did not show obvious cytotoxicity at a concentration nine times higher than the minimal effective concentration (Fig. 3A). The IC_{50} values for PKC were 2.0 nM for **1**, 8.5 nM for **2**, 35 nM for K-252a, 1200 nM for K-252b and 1.2 nM for staurosporine under our assay conditions (Table 5).

Fig. 2. Scanning electron micrograph of spore chains of strain TA-0403 grown on inorganic-salts starch agar for 14 days at 28°C.



Discussion

Several kinds of compounds have been reported to modulate differentiation to nerve cells from precursor cells. The first indolocarbazole-type antibiotic, staurosporine, was shown to be an inducer of neurite outgrowth from PC12 cells.[8] In contrast, K-252a was found to be an inhibitor of NGF-induced neurite outgrowth in PC12 cells.[6,7] After these reports, several synthetic compounds, PD98059,[11] PD90780,[12] AG879[13] were added as inhibitors of NGF-induced neurite outgrowth of PC12 cells. We isolated potent inhibitors from the cultured mycelium identified as *Streptomyces* sp. in our search for this activity. The new compounds were named indocarbazostatin (**1**) and indocarbazostatin B (**2**) on the basis of the structure of their

Table 2. Physiological characteristics of strain TA-0403.

Condition	Characteristic
Temperature range for growth (°C)	16 ~ 40
Optimum temperature for growth (°C)	32 ~ 36
Gelatin liquefaction	-
Milk coagulation	-
Milk peptonization	+
Production of melanoid pigments	-
Starch hydrolysis	+
Carbohydrate utilization:	
D-Glucose	+
L-Arabinose	+
D-Xylose	±
D-Fructose	±
Sucrose	+
L-Rhamnose	+
Raffinose	+
Inositol	+
D-Mannitol	+

+: Positive, ±: weakly positive, -: negative

Table 3. Effects of indocarbazostatin (**1**), indocarbazostatin B (**2**) and K-252a on NGF-induced neurite outgrowth from PC12 cells.

Compound	Minimal effective concentration (nM)
Indocarbazostatin	6
Indocarbazostatin B	24
K-252a	194

Table. 4. Effect of indocarbazostatin (**1**) on PC12 cell viability.

Compound	Viability (%) at MEC[a)]		Viability (%) at MEC x 10[b)]	
	MTT	Calcein/AM	MTT	Calcein/AM
Indocarbazostatin	75	90	35	40
K-252a	70	95	0	0
Cycloheximide (0.1 μM)	0	0		

a) MEC: Minimal effective concentration, indocarbazostatin: 6 nM; K-252a: 200 nM.

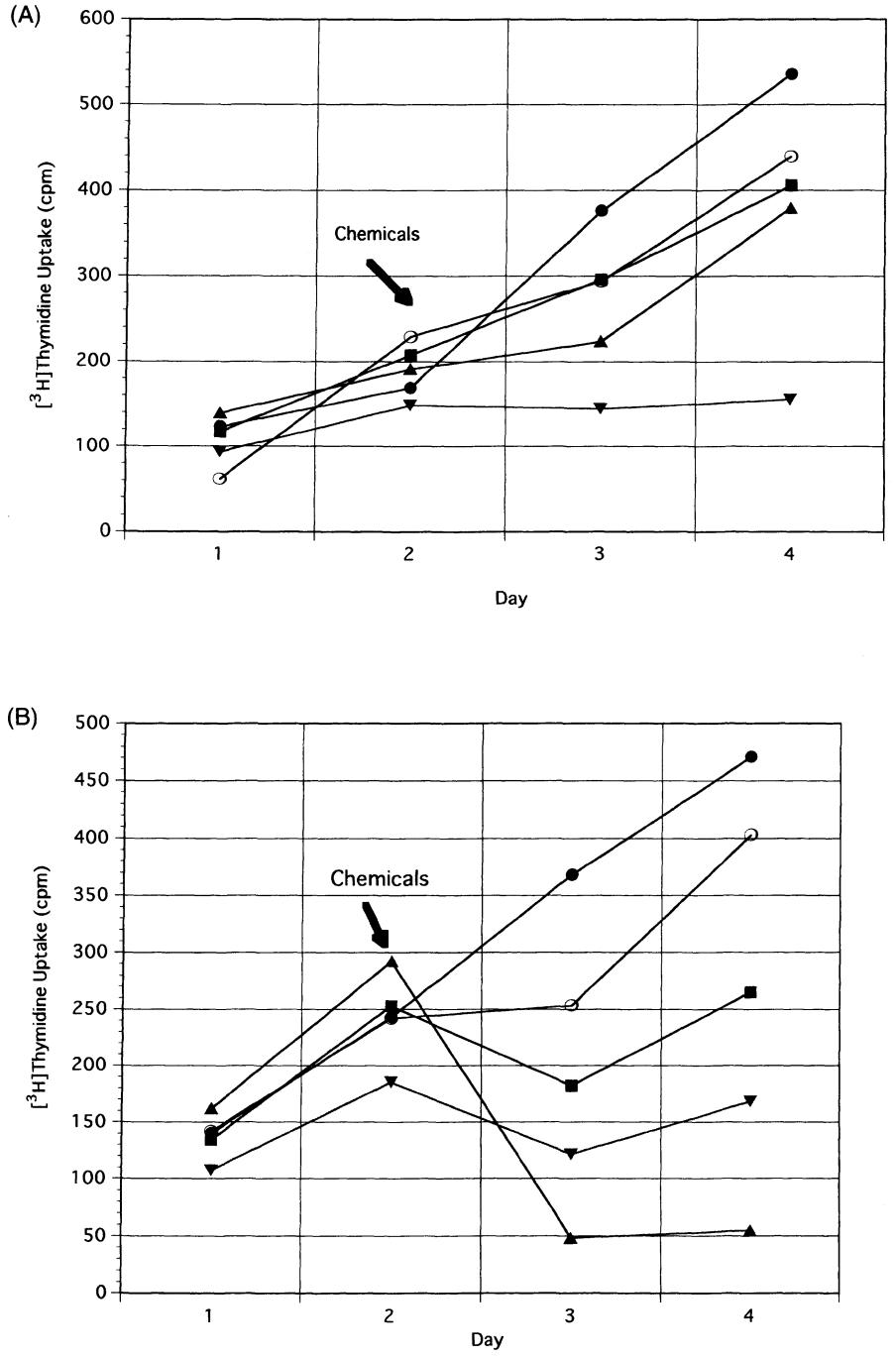
b) MEC x 10: Concentration ten times as high as MEC.

Table 5. Inhibitory activity of indocarbazostatin (**1**) and indocarbazostatin B (**2**) and other indolocarbazole antibiotics against protein kinase C.

Compound	IC_{50} (nM)
Indocarbazostatin (**1**)	2.0
Indocarbazostatin B (**2**)	8.5
K-252a	35
K-252b	1200
Staurosporine	1.2

central chromophore. Compounds **1** and **2** blocked the biological effect of NGF in PC12 cells at 6 nM and 24 nM, respectively. K-252a showed the same activity at 197 nM. This activity could not be observed for K-252b at 197 nM but cytotoxic activity was seen at 388 nM (data not shown). K-252a also showed cytotoxicity at 388 nM under our assay conditions. Both inhibitors **1** and **2** showed cytotoxicity at 97 nM. Selective activity of **1** against the cellular action of NGF was also demonstrated by comparing the cytotoxic data of these compounds by the MTT and Calcein/AM methods as shown in Table 4 and growth inhibition monitored with the incorporation of [^{3}H] thymidine into PC12 cells (Fig. 3A and B). Among these indolocarbazole inhibitors, the order of activity was **1**>**2**>K-252a>K-252b, although K-252b did not show obvious inhibitory activity on neurite outgrowth from PC12 cells under our assay conditions (Table 3). Several indolocarbazole-type antibiotics, including staurosporine, show inhibitory activity against PKC. We, therefore, carried out the kinase assay, and the order of inhibitory activity against PKC was staurosporine >**1**>**2**>K-252a≫K-252b within the tested compounds (Table 5). The strongest inhibitor among these alkaloids is staurosporine (IC_{50}: 1.2 nM), which is comparable to **1** (IC_{50}: 2.0 nM). However, staurosporine induces neurite outgrowth in PC 12 cells at nanomolar concentration in contrast to **1**. RASOULY *et al.* concluded

Fig. 3. Effects of indocarbazostatin (**1**) and K-252a (**3**) on growth of PC12 cells.



A: 0 nM (●), 6 nM (○), 18 nM (■), 54 nM (▲) of indocarbazostatin and 0.1 mM (▼) of cycloheximide.
B: 0 nM (●), 200 nM (○), 600 mM (■), or 1.8 nM (▲) of K-252a and 0.1 mM (▼) of cycloheximide.

that staurosporine-induced neurite outgrowth in PC12 cells is independent of PKC.[8] Since the sugar moieties of **1** and **2** are similar to that of K-252a but not other indocarbazole-type antibiotics, they might block NGF action in the same manner as K-252a. It is reported that K-252a showed the inhibitory activity on NGF-induced neurite outgrowth through inhibition of $p140^{trkA}$, NGF receptor on the cell membrane.[22,23] It remains to be clarified whether the

inhibitory activities on *trk* tyrosine kinase of **1** and **2** are more potent or not than that of K-252a.

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