COMMUNICATIONS TO THE EDITOR

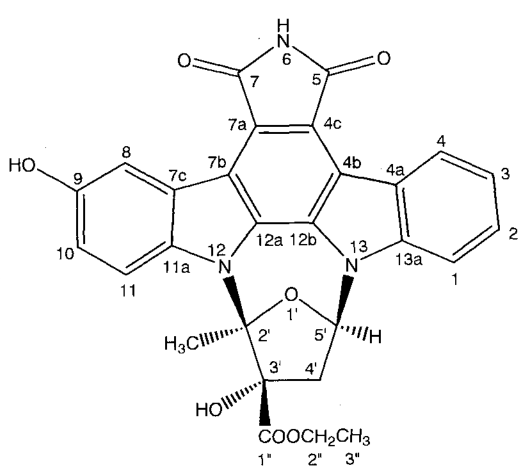
Indocarbazostatin, a Novel Inhibitor of NGF-induced Neurite Outgrowth from Rat Pheochromocytoma PC12 Cells

Sir:

Neurotrophic factors are known to be essential for the normal development and functional maintenance of nerve cells. A decrease in availability of neurotrophic factors is considered to cause dysfunction of the nervous system, resulting in various nerve diseases such as Alzheimer's[1] and Parkinson's[2] diseases and neuronal death induced by brain ischemia.[3] Contrary to this, it was reported that up-regulation of neurotrophic factors are observed in patients with intractable temporal lobe epilepsy[4] and after excitotoxicity in a rat model of Huntington's disease.[5] Therefore, both modulators, inducer and inhibitor, for neurite outgrowth can be useful to treat patients with nerve diseases. Although several modulators, including KS-505a,[6] K252a,[7,8] staurosporine,[9] lactacystin,[10] epolactaene,[11] PD 098059,[12] and AG879[13] have been discovered so far, there are delays in the practical application as therapeutic drugs. Under these circumstance, we started screening for natural substances that regulate differentiation of rat pheochromocytoma PC12 cells. During the course of the screening, an extremely potent inhibitor of NGF-induced neurite outgrowth from PC12 cells, indocarbazostatin, was isolated from a culture broth of a *Streptomyces* sp. (Fig. 1). The compound inhibited NGF-induced neurite outgrowth from PC12 cells at 6 nM, which is approximately 33 times higher than that of K252a[14] in our assay system. In this communication we describe the screening, isolation, structure and biological properties of indocarbazostatin.

Fig. 1. Structure of indocarbazostatin.

The stereochemistry represents relative configuration.



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 D-glucose, 10% fetal bovine serum and 10% horse serum in a 96-well collagen-coated plate, and were treated with test samples after 24 hours. NGF at a final concentration of 50 ng/ml was added 12 hours after sample addition. During a week, the cells were observed under an inverted microscope. The culture extracts and plant extracts which inhibited NGF-induced neurite outgrowth from the cells, were selected. Among 4671 extracts, only one culture, *Streptomyces* sp. TA-0403, was detected, and the isolation of the active substance was followed by the bioassay.

The producing strain identified as *Streptomyces* sp.

Fig. 2. Purification procedure for indocarbazostatin.

Fermentation broth
| centrifuged
Mycelium
| extracted with acetone
Extract
| concentrated *in vacuo*
| extracted with $CHCl_3$
Organic layer
| evaporated *in vacuo*
Crude oil
| silica gel chromatography
| with hexane-acetone (3 : 2)
Active fraction
| HPLC (Mightysil RP-18)
| 65% MeOH
Yellow oil
| crystallized from
| acetone-benzene
Indocarbazostatin

TA-0403 was cultivated in a 500-ml cylindrical flask containing 70 ml of the medium (D-glucose 0.5%, soluble starch 2%, NZ case 0.3%, yeast extracts 0.3%, fish meal 0.5%, $CaCO_3$ 0.2%, pH 6.5) at 30°C for 2 days on a rotary shaker (200 rpm). The seed culture was inoculated into 500-ml cylindrical flasks (500 flasks) containing 70 ml of the same medium, then cultured at 30°C for 3 days. The purification procedure for indocabazostatin is outlined in Fig. 2. The fermentation broth (35 liters) was centrifuged and the mycelial cake was extracted with acetone. After removal of acetone, the aqueous solution was extracted with chloroform. The extracts were subjected to silica gel column chromatography (*n*-hexane - acetone, 3 : 2) and preparative HPLC (Mightysil RP-18, Kanto). Indocarbazostatin was crystallized from acetone-benzene to give 1.7 mg of a pale yellow powder.

It is soluble in acetone, $CHCl_3$, MeOH and DMSO, insoluble in H_2O and *n*-hexane. The molecular formula of indocarbazostatin was determined to be $C_{28}H_{21}N_3O_7$ from the result of HR FAB-MS analysis (Found: *m/z* 512.1439 $(M+H)^+$, Calcd: *m/z* 512.1458). The UV spectrum showed absorption maxima at 236 (ε 4.1×10^4), 283 (ε 2.3×10^4), 290 (ε 2.7×10^4), and 326 nm (ε 4.0×10^6) in MeOH. The IR spectrum (KBr) of indocarbazostatin indicated

Table 1. ^{13}C and ^1H NMR assignments for indocarbazostatin (acetone-d_6)[a].

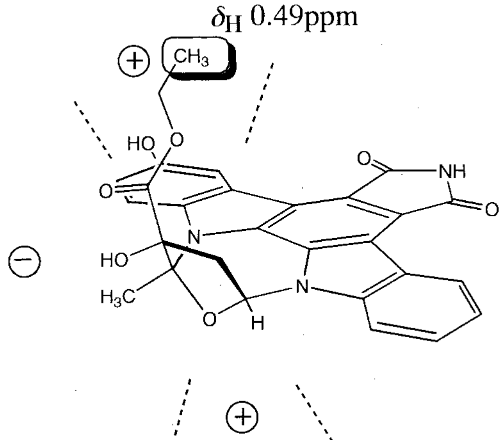
Position	^{13}C	^1H (multiplicity)	J value (Hz)
1	110.5	7.85 (br d)	8.5
2	128.5	7.60 (ddd)	1.2, 7.3, 8.5
3	122.1	7.37 (ddd)	1.2, 7.3, 7.8
4	126.8	9.13 (br d)	7.8
4a	123.5	-	-
4b	118	-	-
4c	ND[c]	-	-
5	ND	-	-
6	-	8.43 (br s)[b]	-
7	ND	-	-
7a	ND	-	-
7b	118.1	-	-
7c	126.2	-	-
8	111.5	8.89 (d)	2.7
9	153.5	9.87 (br s)[b]	-
10	117.5	7.09 (dd)	2.7, 9.0
11	114.7	7.50 (d)	9.0
11a	139.8	-	-
12a	ND	-	-
12b	129.6	-	-
13a	135.2	-	-
2'	104.4	-	-
2'-Me	23.5	2.35 (s)	-
3'	86.3	5.62 (br s)[b]	-
4'	45.2	2.90 (dd); 3.12 (dd)	4.1, 14,9; 7.6, 14.9
5'	87.2	7.30 (dd)	4.1, 7.6
1"	ND	-	-
2"	63.5	3.57 (dq); 3.27 (dq)	10.7, 7.1; 10.7, 7.1
3"	13.7	0.49 (t)	7.1

[a] 100 MHz for ^{13}C NMR and 400 MHz for ^1H NMR.
[b] Exchangeable protons (-NH or -OH).
[c] Not detected.

absorption at 3220, 2980, 1750, 1734, 1700, 1640, 1210 and 960 cm^{-1}. Both UV and IR spectra suggested that the compound possesses a hetero-atom substituted indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6*H*)-dione system as a chromophore.[15~17] The ^1H NMR data in acetone-d_6 of indocarbazostatin, which was compared with the reported data[18] of K252a, are summarized in Table 1. ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, pulse field gradient HMBC, pulse field gradient HMQC spectra revealed the presence of a hydroxy group at the 9 position of the chromophore and a sugar moiety. A quaternary carbon (δ_C 129.6 ppm) could be connected to the sugar moiety through the hetero atom. Observation of a NOE between the methyl proton (δ_H 2.35 ppm) and the aromatic proton (d, 1H, δ_H 7.50 ppm) indicated that the 2′-carbon is bonded to N-12 and 5′ to N-13. The ^1H NMR spectrum of indocarbazostatin showed a methyl proton signal at unusually high magnetic field (3″-H, t, 3H, δ_H 0.33 ppm in $CDCl_3$ and δ_H 0.49 ppm in acetone-d_6) due to the anisotropic effect of the chromophore. The relative configuration of the sugar moiety was deduced by MM2 and MOPAC calculations of the indocarbazostatin molecule. The calculated conformation was similar to that of the ORTEP drawing[18] obtained from X-ray crystallography of K252a except for the opposite configuration at the 3′-position, and the methyl proton at the 3″-position existed in the shielding field of the aromatic chromophore (Fig. 3). Thus, the relative configuration of indocarbazostatin was determined as shown in Fig. 1. Indocarbazostatin is a new member of indolocarbazole-type bioactive molecules.

Indocarbazostatin inhibited NGF-induced neurite outgrowth from PC12 cells at 6 nM, whereas K252a inhibited at 200 nM under our assay conditions (Fig. 4). K252a showed cytotoxicity at a concentration three times higher than the minimal effective concentration; however, indocarbazostatin did not show obvious cytotoxicity to PC12 cells at a concentration nine times higher than the

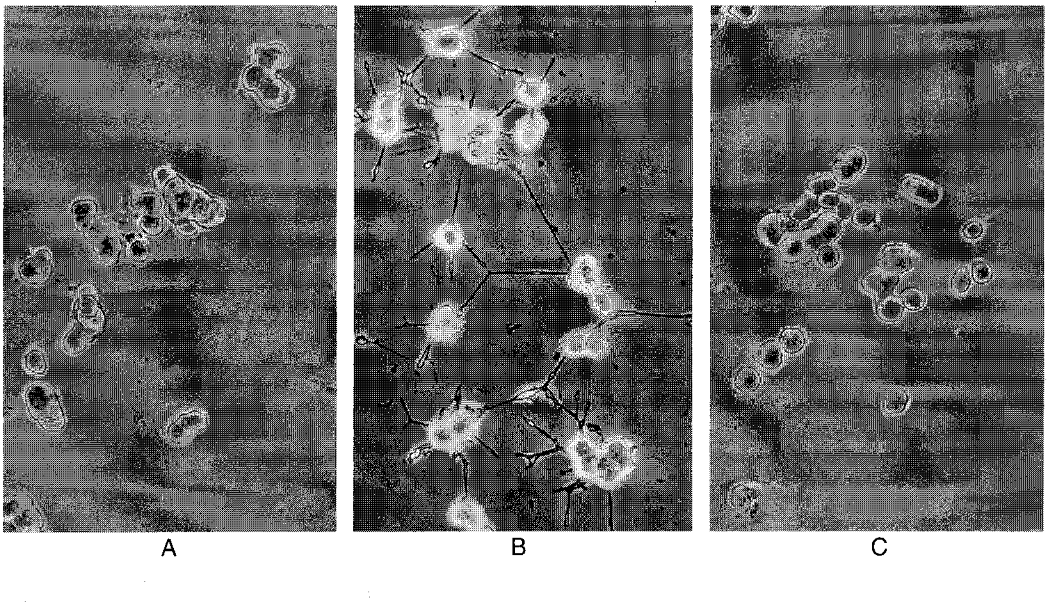
Fig. 3. A stable conformation of indocarbazostatin deduced from MM2 and MOPAC calculation.



The signs, ⊕ and ⊖, denote the shielding and deshielding fields of the aromatic chromophore, respectively.

Fig. 4. Morphological changes of PC12 cells treated with indocarbazostatin in the presence of NGF.

A, control; B, with 50 ng/ml of NGF; C, with 6 nM of indocarbazostatin in the presence of NGF.



minimal effective concentration. The IC_{50} values for protein kinase C from rat brain were; indocarbazostatin 2.0 nM and K-252a 35.0 nM in our assay system.

The isolation and structure elucidation of a minor component, detailed studies on biological and biochemical activities including inhibitory activity against *trk* kinase,[7,8] and the mechanism of action of indocarbazostatin are in progress.

Acknowledgments

The authors are grateful to Dr. A. KAWASHIMA and Mr. T. ANDOH of Taisho Pharmaceutical Co. Ltd. for a gift of the microbial strain and the taxonomic study of the strain. This work was supported, in part, by a Grant-in Aid for Scientific Research (C) provided by the Ministry of Education, Science, Sports and Culture of Japan and a grant for the Biodesign Research Program, the Institute of Physical and Chemical Research (RIKEN).

MAKOTO UBUKATA*
NORIMASA TAMEHIRO
NOBUYASU MATSUURA
NORIYUKI NAKAJIMA

Biotechnology Research Center, Toyama Prefectural University, Kosugi, Toyama 939-0398, Japan

(Received May 13, 1999)

References

1) MUFSON, E. J.; J. S. KROIN, T. J. SENDERA & T. SOBREVIELA: Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. Prog. Neurobiol. 57: 451~485, 1999
2) PARAIN, K.; M. G. MURER, Q. YAN, B. FAUCHEUX, Y. AGID, E. HIRSCH & R. RAISMAN-VOZARI: Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 10: 557~561, 1999
3) MIYAZAKI, H.; Y. OKUMA, Y. FUJII, K. NAGASHIMA & Y. NOMURA: Glial cell-derived neurotrophic factor protects against delayed neuronal death after transient forebrain ischemia in rats. Neuroscience 89: 643~647, 1999
4) TAKAHASHI, M.; S. HAYASHI, A. KAKITA, K. WAKABAYASHI, M. FUKUDA, S. KAMEYAMA, R. TANAKA, H. TAKAHASHI & H. NAWA: Patients with temporal lobe epilepsy show an increase in brain-derived neurotrophic factor protein and its correlation with neuropeptide Y. Brain Res. 818: 579~582, 1999
5) CANALS, J. M.; S. MARCO, N. CHECA, A. MICHELS, E. PEREZ-NAVARRO, E. ARENAS & J. ALBERCH: Differential regulation of the expression of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 after excitotoxicity in a rat model of Huntington's disease. Neurobiol. Dis. 5: 357~364, 1998
6) NAKANISHI, S.; K. OSAWA, Y. SAITO, I. KAWAMOTO, K. KURODA & H. KASE: KS-505a, a novel inhibitor of bovine brain Ca^{2+} and calmodulin-dependent cyclic-nucleotide phosphodiesterase from *Streptomyces argenteolus*. J. Antibiotics 45: 341~347, 1992
7) BERG, M. M.; D. W. STEMBERG, L. P. PARADA & M. V. CHAO: K-252a inhibits nerve growth factor-induced trk proto-oncogene tyrosine phosphorylation and kinase activity. J. Biol. Chem. 267: 13~16, 1992
8) OHMICHI, M.; S. J. DECKER, L. PANG & A. R. SALTIEL: Inhibition of the cellular actions of nerve growth factor by staurosporine and K252a results from the attenuation of the activity of the trk tyrosine kinase. Biochemistry 31: 4034~4039, 1992
9) RASOULY, D.; E. RAHAMIM, D. LESTER, Y. MATSUDA & P. LAZAROVICI: Staurosporine-induced neurite outgrowth in PC12 cells is independent of protein kinase C inhibition. Molecular Pharmacology 42: 35~43, 1992
10) OMURA, S.; T. FUJIMOTO, K. OTOGURO, K. MATSUZAKI, R. MORIGUCHI, H. TANAKA & Y. SASAKI: Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. J. Antibiotics 44: 113~116, 1991
11) KAKEYA, H.; I. TAKAHASHI, G. OKADA, K. ISONO & H. OSADA: Epolactaene, a novel neuritogenic compound in human neuroblastoma cells, produced by a marine fungus. J. Antibiotics 48: 733~735, 1995
12) SPIEGEL, K.; D. AGRAFIOTIS, B. CAPRATHE, R. E. DAVIS, M. R. DICKERSON, J. H. FERGUS, T. W. HEPBURN, J. S. MARKS, M. V. DORF, D. M. WIELAND & J. C. JAEN: PD 90780, a nonpeptide inhibitor of nerve growth factor's binding to the p75 NGF receptor. Biochemica. Biophysica. Res. Commun. 217: 488~494, 1995
13) PANG, L.; T. SAWADA, S. J. DECKER & A. R. SALTIEL: Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. 270: 13585~13588, 1995
14) KASE, H.; K. IWAHASHI & Y. MATSUDA: K-252a, a potent inhibitor of protein kinase C from microbial origin. J. Antibiotics 39: 1059~1065, 1986
15) NETTLETON, D. E.; T. W. DOYLE, B. KRISHNAN, G. K. MATSUMOTO & J. CLARDY: Isolation and structure of rebeccamycin—a new antitumor antibiotic from *Nocardia aerocoligenes*. Tetrahedron Lett. 26: 4011~4014, 1985
16) GOLIK, J.; T. W. DOYLE, B. B. KRISHNAN, G. DUBAY & J. A. MATSON: AT2433-A1, AT2433-A2, AT2433-B1 and AT2433-B2 novel antitumor compounds produced by *Actinomadura melliaura*. II. Structure determination. J. Antibiotics 42: 1784~1789, 1989
17) KOSHINO, H.; H. OSADA & K. ISONO: A new inhibitor of protein kinase C, RK-1409 (7-oxostaurosporine). II. Fermentation, isolation, physico-chemical properties and structure. J. Antibiotics 45: 195~199, 1992
18) YASUZAWA, T.; T. IIDA, M. YOSHIDA, N. HIRAYAMA, M. TAKAHASHI, K. SHIRAHATA & H. SANO: The structures of the novel protein kinase C inhibitors K-252a, b, c and d. J. Antibiotics 39: 1072~1078, 1986