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Indocarbazostatins C and D, New Inhibitors of NGF-induced Neuronal Differentiation in PC12 Cells

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Indocarbazostatins C (**3**) and D (**4**), new inhibitors of NGF-induced neurite outgrowth were isolated from culture broth of a mutant strain, *Streptomyces* sp. MUV-6-83. The structural elucidation of **3** and **4** revealed that these inhibitors were methyl ester analogs of the corresponding ethyl ester compounds, indocarbazostatin (**1**) and indocarbazostatin B (**2**), respectively.

In our screening program designed to discover new inhibitors from microbial products, we reported indocarbazostatin (**1**) and indocarbazostatin B (**2**) as novel inhibitors of NGF-induced neuronal differentiation in PC12 cells.[1~3] To study the biosynthesis of indocarbazostatins, we attempted to obtain high-producing mutants of *Streptomyces* sp. TA-0304[2] that produced **1** and **2**. As a result, we found a high-producing mutant, *Streptomyces* sp. MUV-6-83, which produced new analogs, indocarbazostatins C (**3**) and D (**4**) together with **1** and **2** (Fig. 1). In this paper, we report breeding of the producer of **1** and **2**, production of **3** and **4** by using the mutant, effectiveness of addition of tryptophan and Diaion HP-20 in the production of indocarbazostatins, isolation of **3** and **4**, and biological activity of these molecules.

Materials and Methods

Chemicals

Indocarbazole antibiotic, K-252a, was purchased from CosmoBio Inc. All other chemicals were purchased from Nakarai Tesque (Kyoto, Japan).

Optimal Fermentation Procedure of the Mutant Strain

A slant culture of the mutant strain, *Streptomyces* sp. MUV-6-83 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 (Humko Sheffield Chemical Co.) 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 100 of 500 ml K-1 flask each containing 70 ml of production medium consisting of glucose 0.43%, soluble starch 2.17%, Pharmamedia (Traders Protein) 1.18%, soybean meal 0.59%, corn steep liquor 0.59%, yeast extract (Difco Laboratories) 0.2%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1.0%, D-tryptophan 0.1% (pH 7.0). Fermentation was carried out at 30°C for 4 days on a rotary shaker.

Inhibitory Activities of NGF-induced Neurite Outgrowth of PC12 cells

Inhibitory activities of an NGF-induced biological response, neurite outgrowth of PC12 cells by indocarbazostatins, were measured according to the previously described method.[2] In brief, 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

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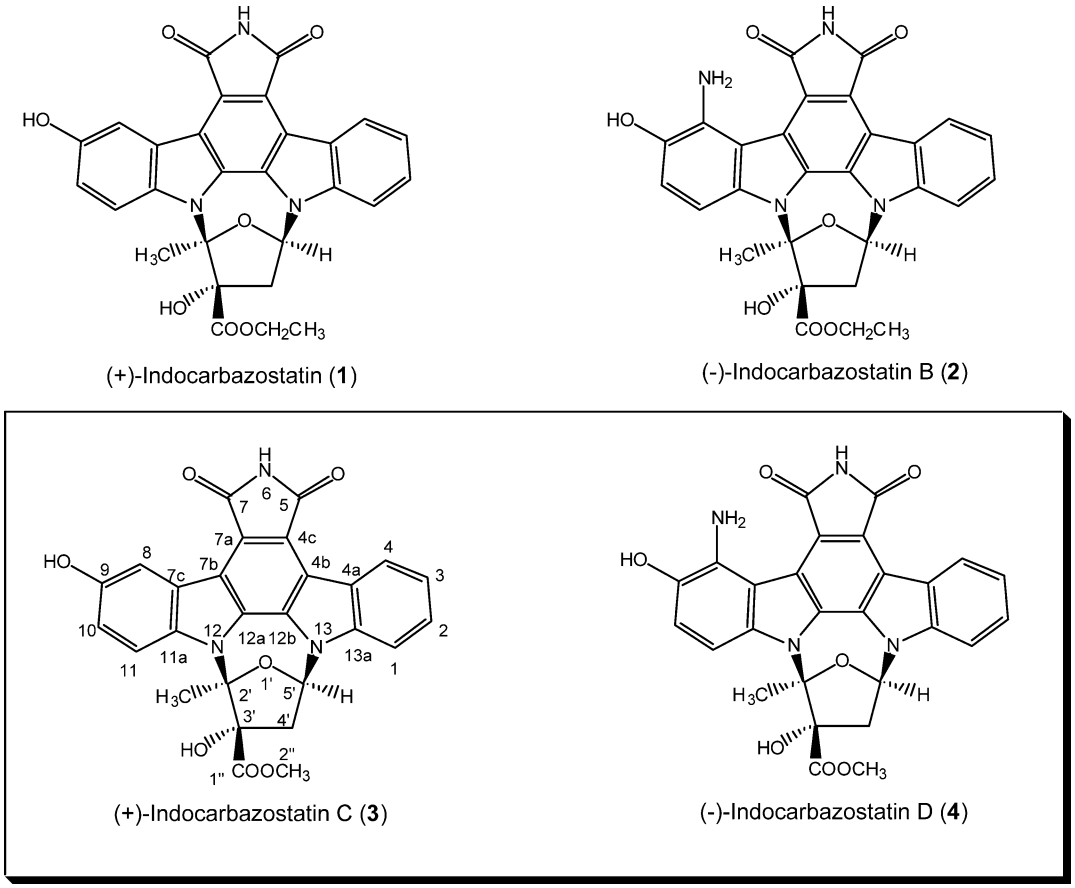
Fig. 1. Structures of indocarbazostatin (**1**), indocarbazostatins B (**2**), C (**3**) and D (**4**).



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, U.S.A.) was added in each well, and effects of the samples on NGF-induced neurite outgrowth were observed under a microscope.

Instruments for Structural Determination

Optical rotation was measured with a Horiba SEPA-300 spectrometer. UV spectra were recorded on a BECKMAN DU-600 spectrophotometer. IR spectra were measured with a SHIMADZU FTIR-8100 spectrometer. NMR spectra were measured on a JEOL JNMEX-270, a JNM-LA400, a Bruker AMX-500 spectrometers, and MS spectra were recorded on a JEOL JMS-AX500 instrument. CD spectra were recorded on a JASCO J-720W spectropolarimeter. Molecular modeling, conformational search with MM2, MOPAC and CONFLEX calculations were performed by using CAChe WorkSystem 4.1 (Oxford Molecular Ltd.) on a Power Macintosh G3.

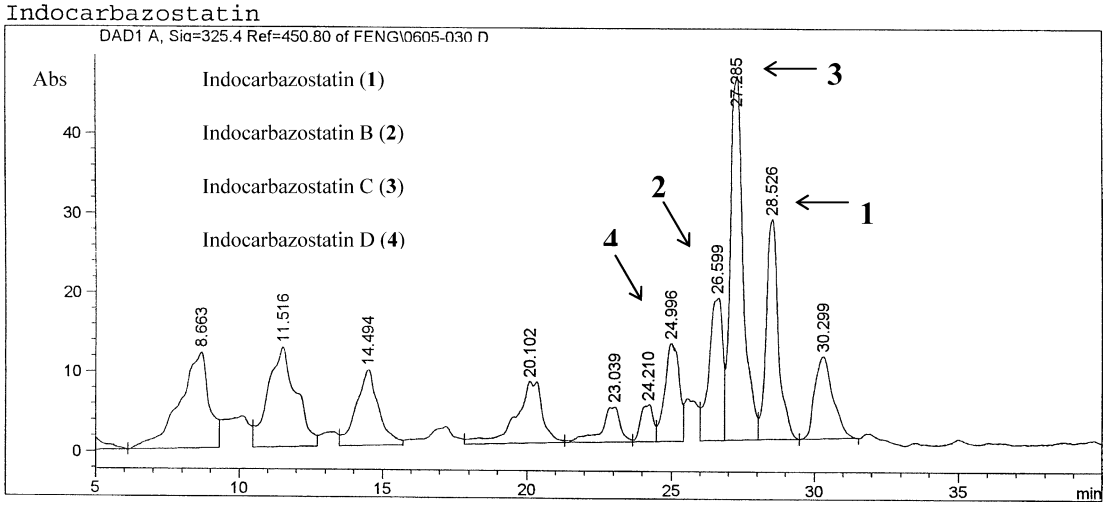
Result and Discussion

Mutation and Production of Indocarbazostatins C (**3**) and D (**4**)

Mutation of the producing strain, *Streptomyces* sp. TA-0403[2] was achieved by irradiating UV light (253.7 nm) for 90 seconds at a distance of 25 cm under a UV lamp. Lethal rate was around 99.95～99.91%. A high-producing mutant was selected after irradiation and mutated again using the same method. After mutation of each isolated mutant was repeated 6 times, we finally obtained a high-producing mutant strain, *Streptomyces* sp. MUV-6-83. The productivity of indocarbazostatin (**1**) and indocarbazostatin B (**2**) was improved 2.5-fold and 8.5-fold, respectively.

We also tested the effectiveness of the addition of tryptophan and Diaion HP-20, since L-tryptophan was

Fig. 2. HPLC Chromatogram of indocarbazostatins produced by the mutant strain, *Streptomyces* sp. MUV-6-83.



reported as a biosynthetic precursor of staurosporin[4] and HP-20 was considered effective for accumulation of indocarbazostatin. In our case, addition of 0.1 % of DL-tryptophan or L-tryptophan greatly enhanced production of indocarbazostatin (**1**) together with indocarbazostatin B (**2**), whereas the addition of D-tryptophan enhanced the production of related new compounds, indocarbazostatins C (**3**) and D (**4**) and also indocarbazostatin (**1**) and indocarbazostatin B (**2**). Furthermore, productivity of indocarbazostatins was successfully promoted by the early addition of HP-20. The optimal fermentation condition for the isolation of **3** and **4** is described in Materials and Methods. An HPLC profile of the $CHCl_3$ extract under the optimized fermentation conditions is shown in Fig. 2.

Isolation of Indocarbazostatins C (**3**) and D (**4**)

The isolation procedure is shown in Fig. 3. A culture broth (7 liters) of *Streptomyces* sp. MUV-6-83 mutant was separated into mycelium and supernatant broth after centrifuging. The mycelium and solids were extracted with acetone, and the extract was concentrated *in vacuo*. To the residue water was added, and the mixture was extracted with the same volume of $CHCl_3$. The organic layer was evaporated *in vacuo* to afford 5.61 g of a crude oil. The crude oil was subjected to silica gel column chromatography with hexane - acetone (3 : 2) as an elution solvent. Indocarbazostatins were monitored by their yellow color on the TLC plate and a similar UV pattern to that of indocarbazostatin on HPLC equipped with a photodiode array detector. The fractions which showed similar UV patterns were concentrated to give 0.7 g of a yellow syrup which was applied to silica gel chromatography with $CHCl_3$ - MeOH (20 : 1) as an elution solvent to give 63 mg of a mixture of indocarbazostatins B (**2**) and D (**4**) and a mixture of 20.4 mg of indocarbazostatin (**1**) and indocarbazostatin C (**3**). Indocarbazostatins B (**2**) and D (**4**) were separated by reversed phase column (Cosmosil 40 C_{18}-PREP, eluent: 70% MeOH) to give 6.7 mg each of crude **2** and **4**, each of which was further purified on reversed phase HPLC (Mightysil RP-18, eluent: 65% MeOH) to yield 2.87 mg of pure indocarbazostatin B (**2**) and 2.68 mg of indocarbazostatin D (**4**), respectively. By similar procedures, 2.48 mg of **1** and 5.18 mg of **3** were isolated.

Physicochemical Properties and Structures

The physicochemical properties of **3** and **4** are summarized in Table 1. The molecular formulae of **3** and **4** were determined to be $C_{27}H_{19}N_3O_7$ and $C_{27}H_{20}N_4O_7$, respectively, by FAB-MS, ^1H and ^{13}C NMR analyses. The ^1H and ^{13}C NMR analyses including NOE experiments of these compounds indicated that **3** and **4** are methyl ester analogs of **1** and **2**, respectively. The C-H long-range coupling patterns in **3** and **4** were obtained as shown in Fig. 4. The NOEs between the C2′-methyl protons and the C11-aromatic protons of **3** and **4** were observed as in the case of

Fig. 3. Isolation procedure of indocarbazostatin (**1**), indocarbazostatins B (**2**), C (**3**) and D (**4**).

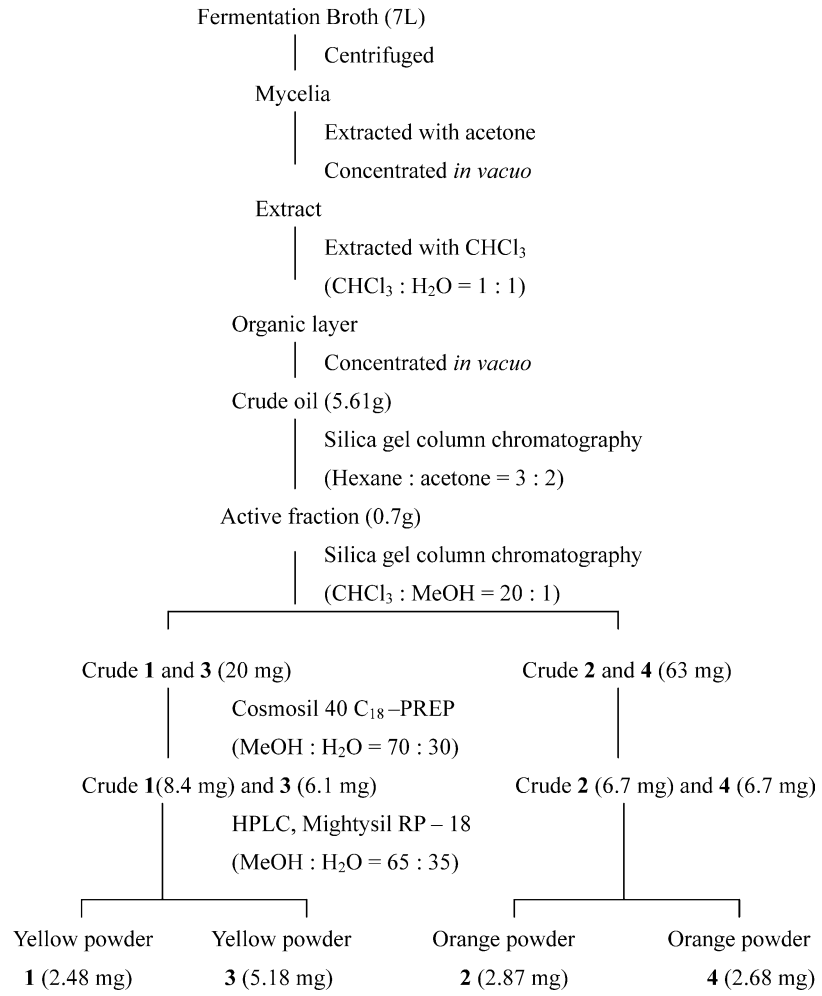
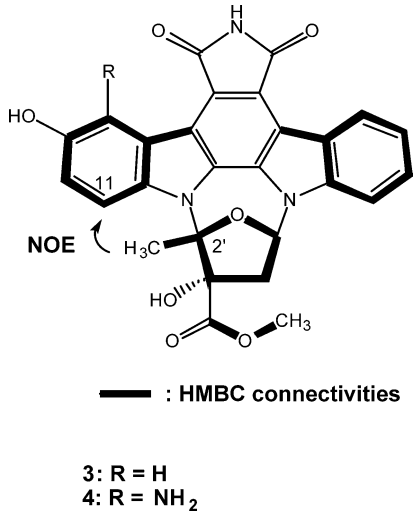


Table 1. Physico-chemical properties of indocarbazostatin C (**3**) and indocarbazostatin D (**4**).

	Indocarbazostatin C (**3**)	Indocarbazostatin D (**4**)
Appearance	yellow powder	orange powder
Molecular formula	$C_{27}H_{19}N_3O_7$	$C_{27}H_{20}N_4O_7$
Molecular weight	497	512
Mp (℃)	259-262	>300
IR νmax (KBr) cm^{-1}	3618(OH),2924(CH) 1734,1714, (COOR,CONHCO),1647(Ph),1269, 1091(COC),796(Ph) ,744	3618(OH),1993,1734,1716 (COOR CONHCO),1647(Ph),1508,1558(NH^+) 1271,993(COC),754
UV $\lambda^{MeOH}{}_{Max}$ nm (log ε)	235.8 (4.44), 286.0 (4.10), 293.0 (4.20),325.4 (4.40)	236.6 (4.06), 269.2 (3.90) 291.4(3.85), 324.8 (3.64)
$[\alpha]^{26}_D$	+ 50.0 (c 0.05,MeOH)	- 44.0 (c 0.05,MeOH)
Solubility	Acetone, $CHCl_3$, MeOH	Acetone, $CHCl_3$, MeOH

1 and **2** (Fig. 4). The ^{1}H and ^{13}C NMR assignments of **3** and **4** are listed in Table 2. The absolute configurations of **3** was found to be the same as that of **1** by the analyses of ^{1}H NMR and an application of the imide/amide sector rule for CD data of indocarbazole antibiotics proposed by the authors.[3] The unusual CD spectrum of **4** was explained by negative atropisomeric chirality (left-handed twist) in the 7b-7c axis, because of repulsion by the steric hindrance between the C-8 NH_2 group and the C-7 carbonyl group as in the case of **2**. The CONFLEX calculation of **4** afforded negative atropisomer in the 7b-7c axis.[3] Both UV and CD spectra of **3** are similar to **1**; and the UV and CD spectra of compounds **2** and **4** are comparable (see Figs. 5 and 6).

Fig. 4. Long-range coupling obtained from HMBC experiments and NOEs between the C2′-methyl protons and the C11-aromatic protons of indocarbazostatins C (**3**) and D (**4**).



Biological Activities

The new compounds, indocarbazostatins C (**3**) and D (**4**), inhibited NGF-induced neurite outgrowth in PC12 cells at 16 and 48 nM respectively, whereas indocarbazostatin (**1**)

Table 2. ^{13}C and ^{1}H NMR assignments for indocarbazostatins C (**3**) and D (**4**) (acetone-d_6)[a].

Indocarbazostatin C (**3**)			
Position	^{13}C	^{1}H multiplicity	J value (Hz)
1	110.0	7.86 (br d)	8.5
2	127.9	7.60 (ddd)	1.2, 7.3, 8.5
3	121.6	7.39 (ddd)	1.2, 7.3, 7.8
4	126.3	9.14 (br d)	7.8
4a	123.0	-	-
4b	117.8	-	-
4c	ND[b]	-	-
5	(171.0)[c]	-	-
6-NH	-	8.46 (br s)	-
7	(171.3)[c]	-	-
7a	ND[b]	-	-
7b	117.5	-	-
7c	125.6	-	-
8	111.0	8.81 (d)	2.2
9	152.9	-	-
9-OH	-	9.89 (br s)	-
10	117.0	7.12 (dd)	2.7, 9.0
11	114.0	7.51 (d)	9.0
11a	134.7	-	-
12a	131.5	-	-
12b	128.7	-	-
13a	139.3	-	-
2’	103.8	-	-
2’-Me	22.9	2.44 (s)	-
3’	85.4	-	-
3’-OH	-	5.70 (br s)	-
4’	44.6	2.90 (dd), 3.15 (dd)	4.4, 14.9, 7.6, 15.0
5’	86.6	7.31 (dd)	4.4, 7.6
1”	171.4	-	-
2”	53.1	3.05 (s)	-

indocarbazostatin D (**4**)			
Position	^{13}C	^{1}H multiplicity	J value (Hz)
1	110.2	7.92 (br d)	8.3
2	128.2	7.64 (ddd)	1.2, 7.3, 8.3
3	122.1	7.40 (ddd)	1.2,7.3,7.8
4	126.5	9.15 (br d)	7.8
4a	122.8	-	-
4b	ND[b]	-	-
4c	ND[b]	-	-
5	(170.2)[c]	-	-
6-NH	-	ND[b]	-
7	(170.5)[c]	-	-
7a	ND[b]	-	-
7b	ND[b]	-	-
7c	117.1	-	-
8	135.2	-	-
9	149.2	-	-
9 -OH	-	9.85 (br s)	-
10	118.9	7.38 (d)	9.4
11	121.3	7.98 (d)	9.4
11a	132.3	-	-
12a	ND[b]	-	-
12b	132.3	-	-
13a	139.2	-	-
2’	104.7	-	-
2’-Me	23.5	2.48 (s)	-
3’	86.0	-	-
3’-OH	-	5.10 (br s)	-
4’	44.7	3.02 (dd), 3.23 (dd)	4.4, 15.1, 7.5, 15.1
5’	86.9	7.379(dd)	4.4, 7.5
1”	171.2	-	-
2”	53.3	3.13 (s)	-

a: 100 MHz for ^{13}C NMR and 400 MHz for ^{1}H NMR.

b: Not detected. c: Assignments may be interchangeable.

Fig. 5. Comparison of UV spectra of indocarbazostatins C (**3**) and D (**4**) with indocarbazostatin (**1**) and indocarbazostatin B (**2**).

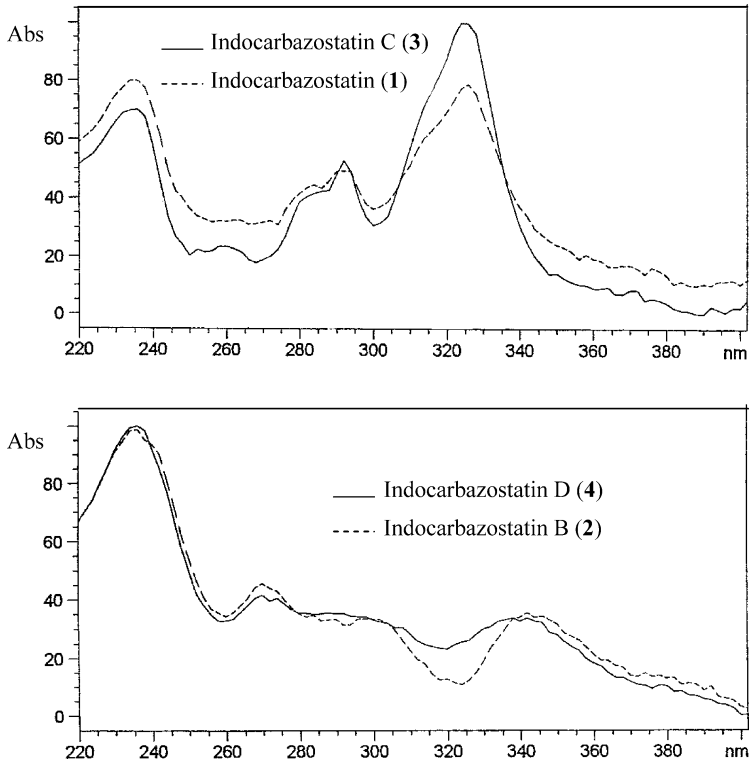
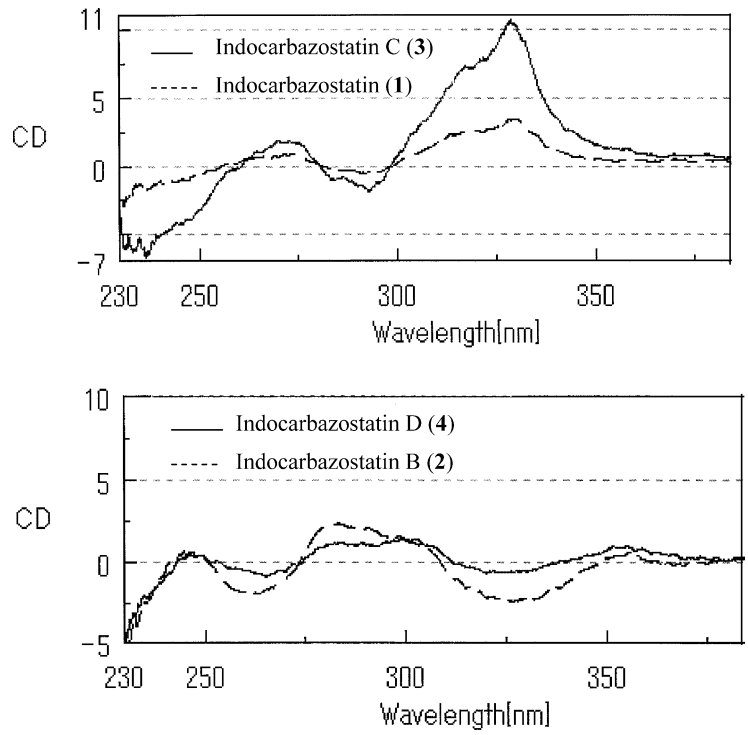


Fig. 6. Comparison of CD spectra of indocarbazostatins C (**3**) and D (**4**) with indocarbazostatin (**1**) and indocarbazostatin B (**2**).



Cell length: 0.1 cm; concentration: 0.1 mg/ml; solvent: $CHCl_3$.

Table 3. Inhibitory effects of indocarbazostatins on neurite outgrowth in PC12 cells.

Compound	Minimal effective concentration (nM)
	Inhibitory dose for NGF-induced neurite outgrowth in PC 12 cells
Indocarbazostatin (**1**)	3
Indocarbazostatin B (**2**)	80
Indocarbazostatin C (**3**)	16
Indocarbazostatin D (**4**)	48
K252a	80

and indocarbazostatin B (**2**) inhibited at 3 and 80 nM respectively under the experimental conditions (Table 3). The most effective inhibitor was **1**, followed by **3**; both containing the 8-NH_2, whereas analogs, **2** and **4**, showed rather weak activities. However, methyl analog (**4**) was more effective than ethyl analog (**2**) under these experimental conditions. Recently, GRINGRICH *et al.* reported the synthesis and kinase inhibitory activity of 3′-(*S*)-epi-K-252a and led to the conclusion that inverting the 3′-alcohol resulted in enhancement of inhibitory activity on VEGFR2 and TrkA tyrosine kinase.[5] These findings suggest that the potent activities of **1** and **3** are partially explained by the opposite configuration at C3′ of indocarbazostatins to that of K-252a.[6,7] Thus, potent biological activities of **1** and **3** may be related to the inhibition of the trk tyrosine kinase as in the case of K-252a.[8~10] The existence of amino groups and strained chromophore structures in **2** and **4** reduced the inhibitory activity against neurite outgrowth of PC12 cells induced by NGF.

In conclusion, attempts to enhance productivity of indocarbazostatins by inducing mutation to the original producer, *Streptomyces* sp. TA-0403, with UV irradiation led to the acquirement of a high-producing mutant that produced new analogs, indocarbazostatins C (**3**) and D (**4**). The addition of D-tryptophan and Diaion HP-20 was effective for the enhanced production of such new compounds. Indocarbazostatins C (**3**) and D (**4**) were found to be methyl ester analogs of indocarbazostatin (**1**) and indocarbazostatin B (**2**). To confirm that such methyl esters are not artifacts, we checked the productivity of **3** and **4** under similar conditions without using methanol. All indocarbazostatins **1**, **2**, **3** and **4** were detected on HPLC under these conditions. Thus, indocarbazostatins C (**3**) and D (**4**) are natural products produced *via* a shunt pathway of biosynthesis of indocarbazostatin (**1**) and indocarbazostatin B (**2**).

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References

1) UBUKATA, M.; N. TAMEHIRO, N. MATSUURA & N. NAKAJIMA: Indocarbazostatin, a novel inhibitor of NGF-induced neurite outgrowth from rat pheochromocytoma PC12 cells. J. Antibiotics 52: 921～924, 1999
2) MATSUURA, N.; N. TAMEHIRO, T. ANDOH, A. KAWASHIMA & M. UBUKATA: Indocarbazostatin and indocarbazostatinB, novel inhibitors of NGF-induced neuronal differentiation in PC12 cells. I. Screening, taxonomy, fermentation and biological activities. J. Antibiotics 55: 355～362, 2002
3) TAMEHIRO, N.; N. MATSUURA, Y. FENG, N. NAKAJIMA & M. UBUKATA: Indocarbazostatin and indocarbazostatin B, novel inhibitors of NGF-induced neuronal differentiation in PC12 cells. II. Isolation, physicochemical properties and structural elucidation. J. Antibiotics 55: 363～370, 2002
4) MEKSURIYEN, D. & G. A. CORDELL: Biosynthesis of staurosporine, 2. Incorporation of tryptophan. J. Natural Products 51: 893～899, 1988
5) GINGRICH, D. E. & R. L. HUDKINS: Synthesis and kinase inhibitory activity of 3′-(*S*)-epi-K-252a. BioMed. Chem. Lett. 12: 2829～2831, 2002
6) KASE, H.; K. IWAHASHI & Y. MATSUDA: K-252a, a potent inhibitor of protein kinase C from microbial origin. J. Antibiotics 39: 1059～1065, 1986
7) YASUZAWA, T.; T. IIDA, M. YOSHIDA, N. HIRAYAMA, M. TAKAHASHI, K. SHIRAHATA & H. SANO: The structure of the novel protein kinase C inhibitors K-252a, b, c and d. J. Antibiotics 39: 1072～1078, 1986
8) BERG, M. M.; D. W. STERNBERG, L. F. PARADA & M. V. CHAO: K-252a inhibits nerve growth factor-induced trk proto-oncogene tyrosine phosphorylation and kinase activity. J. Bio. Chem. 267: 13～16, 1992
9) TAPLEY, P.; F. LAMBALLE & M. BARBACID: K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. Oncogene 7: 371～381, 1992
10) 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