VOL. 55 NO. 4, APR. 2002 THE JOURNAL OF ANTIBIOTICS pp. 363 — 370

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

II. Isolation, Physicochemical Properties and Structural Elucidation

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(Received for publication October 16, 2001)

Two novel indolocarbazol-type bioactive molecules, indocarbazostatin (**1**) and indocarbazostatin B (**2**), were isolated as inhibitors of NGF-induced neuronal differentiation in rat pheochromocytoma PC12 cells from a culture broth of a *Streptomyces* sp. The structures of these compounds were determined by HR-FAB-MS, UV, ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, PFG HMBC, PFG HMQC and DIF NOE experiments. The relative and absolute configurations were deduced from MM2, MOPAC and CONFLEX calculations, and CD analyses. The imide/amide sector rule was proposed from the analyses of CD data of **1** and other indolocarbazole antibiotics. It was concluded that the minor compound **2** has a negative atropisomeric chirality in the aglycone.

During the course of our screening for modulators of signal transduction of mammalian cells, we discovered two novel inhibitors, indocarbazostatin (**1**) and indocarbazostatin B (**2**) from the culture broth of a *Streptomyces* sp. TA-0403 (Fig. 1). In our assay system, **1** inhibited NGF-induced neurite outgrowth in PC12 cells at 6 nM, which is approximately 33 times lower than the minimal effective concentration of K252a.[1,2] In this paper, we report details of isolation, physicochemical properties and structural elucidation of these inhibitors. Taxonomy and fermentation procedures of the producing microorganisms, and biological activities of these compounds are described in the preceding paper.[3]

Results and Discussion

Isolation of Indocarbazostatin and Indocarbazostatin B

The purification procedure of **1** and **2** is shown in Fig. 2. A culture broth (35 liters) of *Streptomyces* sp. TA-0403 strain was separated into mycelium and supernatant broth after centrifuging. The mycelium was extracted with acetone, and the extract was concentrated *in vacuo*, water was added, and the mixture was extracted with $CHCl_3$. The organic layer was evaporated *in vacuo* to give a crude oil. This residue was applied to silica gel column chromatography with hexane - acetone (3 : 2) as an elution solvent. The active fractions were concentrated to give a syrup which was applied to reversed phase HPLC (Mightysil RP-18, 65% MeOH) to yield 4 mg of a pale yellow syrup as the major active component showing retention time at 29 minutes and 1.2 mg of a pale yellow syrup as the minor component showing retention time at 28 minutes. The major compound was crystallized from acetone-benzene to give 1.7 mg of indocarbazostatin (**1**). The minor compound was cautiously crystallized from acetone-benzene to give 0.9 mg of indocarbazostatin B (**2**).

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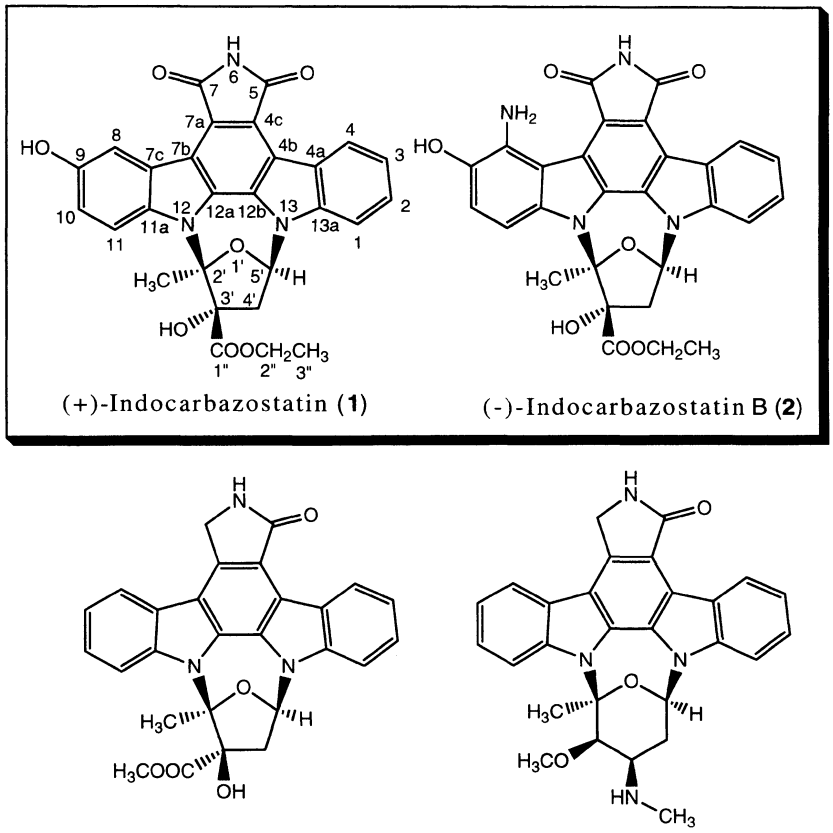
Fig. 1. Structures of indocarbazostatin (**1**), indocarbazostatin B (**2**), K252a (**3**) and staurosporine (**4**).



Physicochemical Properties and Structures

In our preliminary communication,[4] we reported the relative configuration of indocarbazostatin (**1**), which differs from K-252a (**3**)[5] in terms of the C-7 carbonyl group, the C-9 hydroxyl group, the ethyl ester group at C-1″ carbonyl and the relative configuration at position C-3′. In this paper, we report further details and the absolute configurations of **1** and **2**.

The physicochemical properties of **1** and **2** are summarized in Table 1. The molecular formula of **1** was determined to be $C_{28}H_{21}N_3O_7$ by high-resolution FAB-MS and ^{13}C NMR analyses. It was found that **1** possesses a hetero-substituted indo[2,2-a]-pyrrolo[3,4-*c*]carbazole-5,7(6H)-dione system as its chromophore by the analyses of ^1H and ^{13}C NMR, and ^1H-^1H COSY, pulse field gradient HMQC (PFG HMQC), pulse field gradient HMBC (PFG HMBC) and differential NOE (DIF NOE) data as well as IR and UV absorption data, and by ZINDO calculation of the electronic spectrum. The ^1H and ^{13}C NMR assignments of **1** are listed in Table 2. ^1H and ^{13}C NMR, ^1H-^1H COSY, PFG HMQC and PFG HMBC analyses revealed the presence of a hydroxyl group at C-9 of the chromophore and a sugar moiety. A quaternary carbon (δ_C 129.6 ppm) is connected to the sugar moiety *via* the nitrogen atom. Long-range couplings obtained from an HMBC experiment are shown in Fig. 3. Observation of an NOE between the methyl proton (δ_H 2.35 ppm) and the aromatic proton (d, 1H, δ_H 7.50 ppm) indicated that C-2′ is bonded to N-12, and C-5′ is bonded to N-13 (Fig. 3). The ^1H NMR spectrum of **1** showed ethyl ester proton signals with unusually low δ_H-value (2″-H, each dq, each 1H, δ_H 3.27 and 3.57; 3″-H, t, 3H, δ_H 0.49 ppm in acetone-d_6) due to the anisotropic effect of the chromophore. The relative configuration of the sugar in **1** was deduced by MM2 and MOPAC calculations. The calculated conformation was similar to that of the ORTEP drawing obtained from the X-ray crystallography of K-252a[5] except for the opposite

Fig. 2. Isolation procedures of indocarbazostatin (**1**) and indocarbazostatin B (**2**).

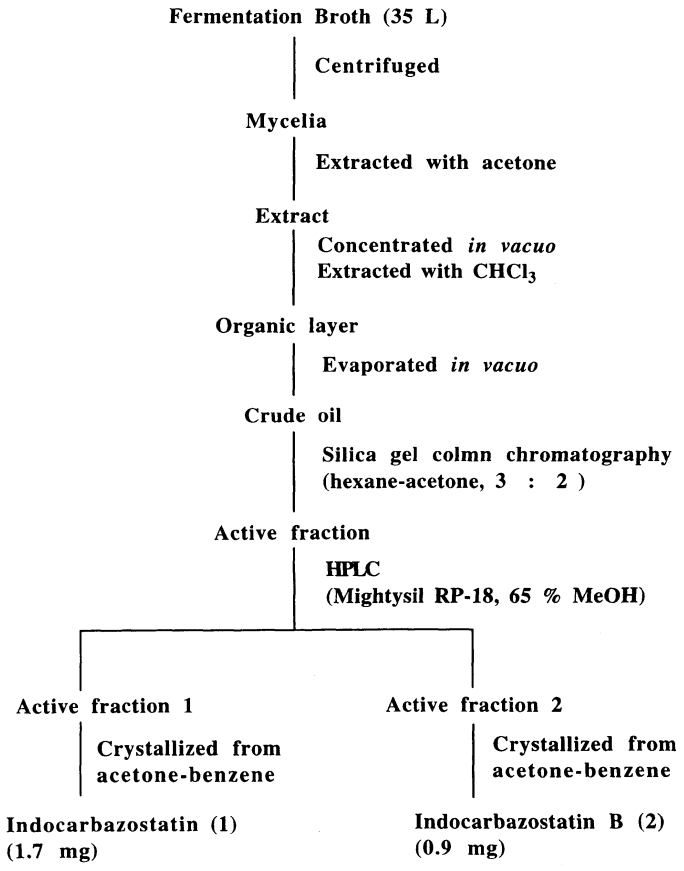


Table 1. Physico-chemical properties of indocarbazostatin (**1**) and indocarbazostatin B (**2**).

	Indocarbazostatin (**1**)	Indocarbazostatin B (**2**)
Appearance	Yellow powder	Yellow powder
Molecular formula	$C_{28}H_{21}N_3O_7$	$C_{28}H_{22}N_4O_7$
Molecular weight	511	526
Mp (°C)	256 (dec)	255 (dec)
IR υ max (KBr) cm^{-1}	3290 (OH), 2920 (CH), 1995,1740, 1710 (COOR, CONHCO), 1640 (Ph), 1260, 1100 (COC), 800 (Ph), 750	3610 (OH), 2960, 2920(CH, NH_3^+), 1995,1740,1710 (COOR, CONHCO), 1640 (Ph), 1510, 1560 (NH_3^+), 1275, 990 (COC), 800(Ph), 755
UV λ^{MeOH}_{Max}nm (log ε)	236 (4.61), 283 (3.36), 290 (4.43), 326 (4.60)	236 (4.36), 270 (4.11), 292 (4.04), 327 (4.04)
$[\alpha]^{26}_D$	+ 51.3 (*c* 0.05, MeOH)	- 48.7 (*c* 0.05, MeOH)
Solubility	Acetone, $CHCl_3$, MeOH	Acetone, $CHCl_3$, MeOH

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

Position	^{13}C	^{1}H (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[b]	-	-
5	ND	-	-
6-NH	-	8.43 (br s)	-
7	ND	-	-
7a	ND	-	-
7b	118.1	-	-
7c	126.2	-	-
8	111.5	8.79 (d)	2.7
9	153.5	-	-
9-OH	-	9.87 (br s)	-
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	-	-
3'-OH	-	5.62 (br s)	-
4'	45.2	2.90 (dd), 3.12 (dd)	4.4, 14.9; 7.6, 14.9
5'	87.2	7.30 (dd)	4.1, 7.6
1"	ND	-	-
2"	63.5	3.27 (dq), 3.57 (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 400MHz for ^{1}H NMR.

b: Not detected.

Fig. 3. Long-range couplings obtained from HMBC experiments and NOEs between the C2′-methyl protons and the C11-aromatic protons of indocarbazostatin (**1**) and indocarbazostatin B (**2**).

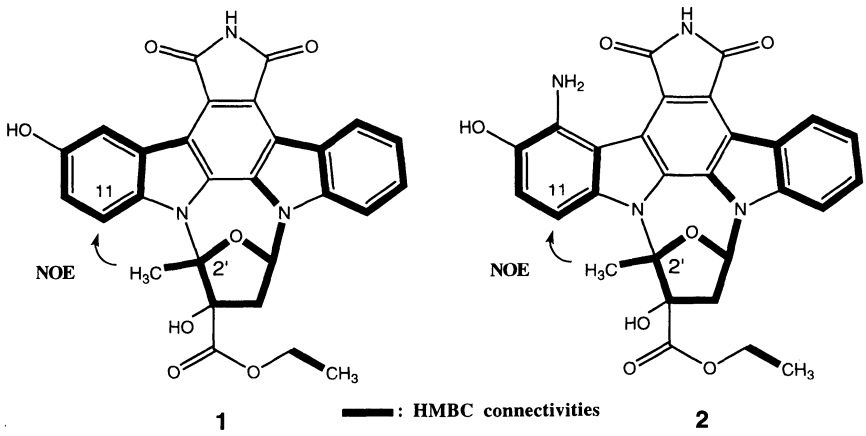


Fig. 4. Relative configurations of indocarbazostatin (**1**) and K-252a (**3**) explain the unusual chemical shifts of the methyl protons.

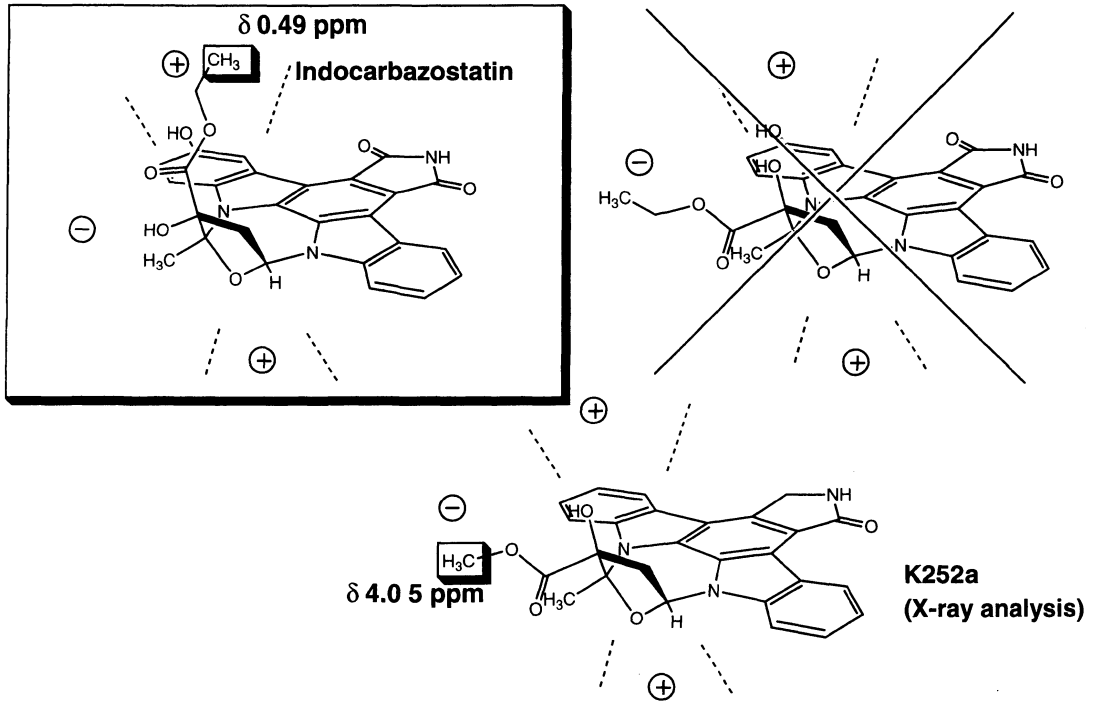
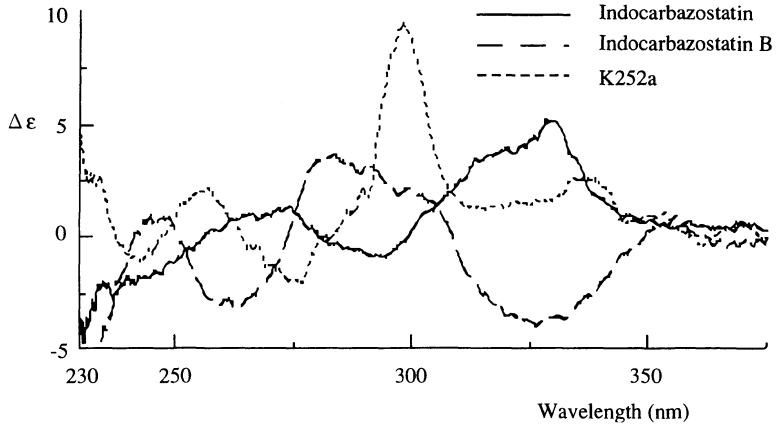


Fig. 5. CD spectra of indocarbazostatin (**1**), indocarbazostatin B (**2**), and K-252a (**3**) in $CHCl_3$.



configuration at the C-3′ asymmetric carbon existing in the deshielding field of the aromatic chromophore, as shown in Fig. 4. These observations reasonably explained why the ^1H NMR spectra of **1** and **2** showed an ester ethyl signal with an unusually high-field chemical shift, in contrast to the ^1H NMR spectrum of K-252a that showed an ester methyl signal with a low-field chemical shift.[5] To determine the absolute configuration, we measured the CD spectra of **1**, and found a positive Cotton effect at 329 nm (Fig. 5). An extension of the lactone sector rule[6] revealed that **1** has the same absolute configuration as K-252a at positions C-2′ and C-5′. The molecule was viewed from the line on the plane of the imide group along the bisectrix of the O=C–N angle, *i.e.*, the line from C-5 to C-5′ as shown in Fig. 6. The

Fig. 6. Application of an imide sector rule to indocarbazostatin (**1**).

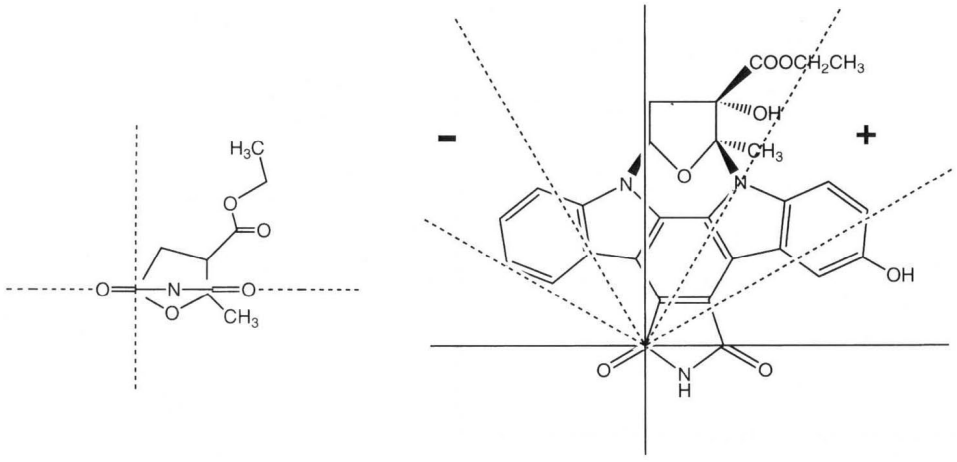
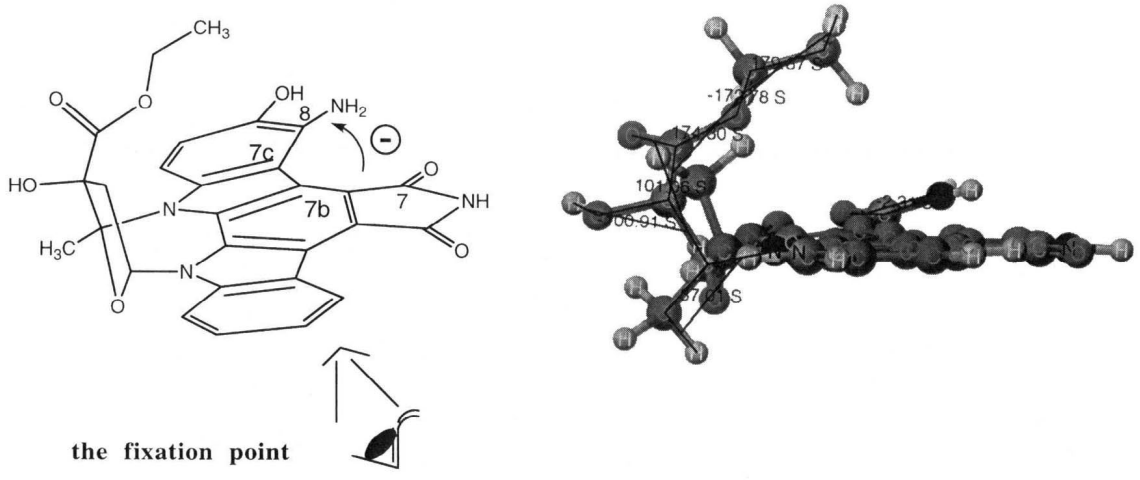


Fig. 7. Side-on view of the energy-minimized structure of indocarbazostatin B (**2**) and its negative atropisomeric chirality in the 7b~7c axis.



functional group at C-3′ lying in the back upper right sector was responsible for the positive Cotton effect. The C–N bond was assumed to have a double-bond character, and as a crude approximation, we assumed that the O=C and C–N bonds are equivalent, and that the plane bisecting the carboxyl angle (O=C–N) is asymmetric. Thus, the ethyl ester group at C-3′ falls within the strongly positive sector (Fig. 6). A similar treatment in which the molecule was viewed along the bisectrix of the C-7 N–C=O angle showed no atom of **1** within the positive or negative sector. Thus, the total structure of **1** was determined, and is shown in Fig. 1. This treatment enabled us to explain satisfactorily the signs of the contributions made by atoms and groups in the indolocarbazole antibiotics possessing an amide, K-252a (Fig. 5), staurosporine (**4**) and RK-286C,[7] all of which showed positive first Cotton effects in their CD spectra, and their absolute configurations have also been proven synthetically.[8~10] The imide/amide sector rule proposed here may be useful for the determination of the absolute configuration of new indolocarbazole antibiotics and other imide or amide compounds in combination with their conformational analyses.

Table 3. ^{13}C and ^{1}H NMR assignments for indocarbazostatin B (**2**) ($CDCl_3$)[a].

Position	^{13}C	^{1}H (multiplicity)	J value (Hz)
1	108.2	7.55 (br d)	8.5
2	127.8	7.65 (ddd)	8.0, 1.1, 8.1
3	121.9	7.47 (br t)	8.1
4	126.4	9.17 (br d)	8.1
4a	121.2	-	-
4b	118	-	-
4c	(135.4)[b]	-	-
5	(168.7)[c]	-	-
6	-	ND[d]	-
7	(168.9)[c]	-	-
7a	(135.5)[b]	-	-
7b	(117.8)	-	-
7c	116.5	-	-
8	134.0	-	-
9	149.2	-	-
10	118	7.27 (d)	9.2
11	120	7.69 (d)	9.2
11a	130.9	-	-
12a	(131.4)	-	-
12b	127.7	-	-
13a	138.1	-	-
2'	103	-	-
2'-Me	23.1	-	-
3'	84.4	-	-
4'	43.3	2.96 (dd), 3.11 (dd)	4.4, 15.3; 7.7, 15.3
5'	85.7	7.08 (dd)	4.4, 7.3
1"	171.3	-	-
2"	64.0	3.18 (dq), 3.71 (dq)	11, 7.0; 11, 7.0
3"	12.7	0.41 (t)	7.0

a: 150 MHz for ^{13}C NMR and 600 MHz for ^{1}H NMR.
b and *c:* Assignments may be interchangeable.
d: Not detected.

The structure of the minor component, **2** was obtained through a similar approach. The molecular formula of **2** was determined to be $C_{28}H_{22}N_4O_7$ by high-resolution FAB-MS and ^{13}C NMR analyses. It is found that **2** is a simple derivative in which H at position C-8 was substituted by the NH_2 group, deduced from the data of UV, IR, ^{1}H and ^{13}C NMR, DIF NOE experiment and ^{1}H-^{1}H COSY, PFG HMQC, PFG HMBC, ^{13}C NMR chemical shift calculation and biosynthetic consideration. The biosynthetic consideration would give the conclusion that **2** has the same absolute configuration as **1**. However, **2** shows an opposite $[\alpha]_D$ value, and the CD spectrum of **2** is totally different from that of **1** as shown in Fig. 5. To elucidate the unusual CD spectrum of **2**, we performed CONFLEX[11,12] calculations of the molecule. The results showed that **2** has 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 C7 carbonyl group (Fig. 7). This observation explains the unusual negative sign shown in the CD spectrum of **2** which has the same

absolute configuration as **1**. Thus, we propose the total structure of **2** as depicted in Fig. 1. The ^{1}H and ^{13}C NMR assignments for **2** are listed in Table 3.

Experimental

General

Optical rotations were measured with a High Sensitive Polarimeter SEPA-300. UV spectra were recorded on a BECKMAN DU-600 spectrophotometer. IR spectra were measured with a SHIMADZU FTIR-8100. FAB MS spectra were measured with a JEOL JMS-AX 500 Mass spectrometer. CD spectra were recorded on a JASCO J-720W spectropolarimeter. NMR spectra were recorded on a JEOL Lambda 400 spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, and a JEOL alpha 600 spectrometer at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. Molecular modeling, conformational search with MM2, MOPAC and CONFLEX calculations and generation of the electronic spectra with ZINDO calculations were performed by using CAChe WorkSystem 4.1 (Oxford Molecular Ltd.) on a Power Macintosh G3.

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 their support. We also thank Dr. H. KOSHINO of the Institute of Physical and Chemical Research (RIKEN) for NMR measurements of **2** with 600 MHz NMR instrument. This work was supported, in part, by a Grant-in Aid for Scientific Research provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1) HASHIMOTO, S.: K-252a, a potent protein kinase inhibitor, blocks nerve growth-induced neurite outgrowth and changes in the phosphorylation of proteins in PC12 cells. J. Cell. Biol. 107: 1531~1539, 1988
2) KOIZUMI, S.; M. L. CONTRERARAS, Y. MATSUDA, T. HAMA, P. LAZAROVICI & G. GUROFF: K-252a: A specific inhibitor of the action of nerve growth factor on PC12 cells. J. Neurosci. 8: 715~721, 1988
3) MATSUURA, N.; N. TAMEHIRO, T. ANDOH, A. KAWASHIMA & M. UBUKATA: Indocarbazostatin and indocarbazostatin B, novel inhibitors of the differentiation of PC-12 cells induced by nerve growth factor. I. Screenig, taxonomy, fermentation and biological activities. J. Antibiotics 55: 355~362, 2002
4) 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
5) 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
6) JENNINGS, J. P.; W. KLYNE & P. M. SCOPES: Optical rotatory dispersion. Part XXIV. Lactones. J. Chem. Soc. 7211~7229, 1965
7) TAKAHASHI, T.; H. OSADA, M. URAMOTO & K. ISONO: A new inhibitor of protein kinase C, RK-286C (4′-demethylamino-4′-hydoroxy-staurosporine) II. Isolation, physico-chemical properties and structure. J. Antibiotics 43: 168~173, 1990
8) WOOD, J. L.; B. M. STOLTZ, H. J. DIETRICH: Total synthesis of (+)- and (−)-K252a. J. Am. Chem. Soc. 117: 10413~10414, 1995
9) LINKS, J. T.; S. RAGHAVAN, M. GALLANT, S. J. DANISHEFSKY, T. C. CHOU & L. M. BALLAS: Staurosporine and ent-staurosporine: The first total syntheses for a regioselective approach, and activity profiles. J. Am. Chem. Soc. 118: 2825~2842, 1996
10) WOOD, J. L.; B. M. STOLTZ, S. N. GOODMAN & K. ONWUEME: Design and implementation of an efficient synthetic approach to pyranosylated indolocarbazoles: total synthesis of (+)-RK286c, (+)-MLR-52, (+)-staurosporine, and (−)TAN-1030a. J. Am. Chem. Soc. 119: 9652~9661, 1997
11) GOTO, H. & E. OSAWA: Corner flapping: a simple and fast algorithm for exhaustive generation of ring conformations. J. Am. Chem. Soc. 111: 8950~8951, 1989
12) GOTO, H. & E. OSAWA: An efficient algorithm for searching low-energy conformers of cyclic and acyclic molecules. J. Chem. Soc. Perkin Trans. 2: 187~198, 1993