A NEW INHIBITOR OF PROTEIN KINASE C, RK-1409B (4'-DEMETHYLAMINO-4'-HYDROXY-3'-EPISTAUROSPORINE)

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RK-1409B, a new inhibitor of protein kinase C, was isolated from the culture broth of *Streptomyces platensis* subsp. *malvinus* RK-1409. The structure was elucidated on the basis of spectroscopic analyses. RK-1409B inhibited protein kinase C *in vitro* and the morphological change of a human chronic leukemia cell line, K-562, induced by phorbol 12,13-dibutyrate with IC_{50} value of 0.4 μM.

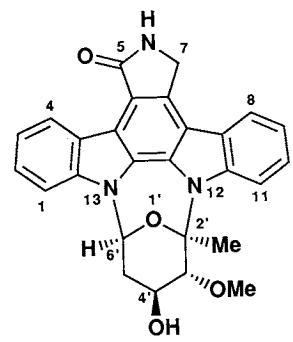
Protein kinase C (PKC) has a crucial role in signal transduction, cellular regulation, proliferation, and tumor promotion[1,2]. Specific inhibitors of PKC were expected to be excellent tools for understanding the roles of PKC. Previously, we reported the PKC inhibitors, sangivamycin[3], RK-286C[4], RK-286D[5], and RK-1409 (7-oxostaurosporine)[6,7] by using a convenient assay system for PKC inhibitors, named bleb forming assay[8]. This assay is based on the morphological change of a human chronic leukemia cell line K-562. We found that *Streptomyces platensis* subsp. *malvinus* RK-1409, the RK-1409 (7-oxostaurosporine)-producing strain[6], produced an additional novel inhibitor of PKC, RK-1409B. In this paper, we report isolation, physico-chemical properties, structural elucidation and biological activities of the novel indolocarbazole antibiotic RK-1409B.

Experimental

General

MP was measured with a Yanaco micro melting point apparatus. Optical rotation and CD spectrum were measured by a Perkin-Elmer 241MC polarimeter and a Jasco J-20A spectropolarimeter, respectively. UV and IR spectra were taken on a Hitachi 220A spectrometer and a Shimadzu IR27G recording IR spectrometer, respectively. EI-MS spectra were obtained with a Hitachi M-80 mass spectrometer. NMR spectra were recorded on a Jeol GX-400 and GSX-500 spectrometers.

Fig. 1. Structure of RK-1409B.



Fermentation

Streptomyces platensis subsp. *malvinus* RK-1409 was cultured in two 500-ml cylindrical flasks containing 140 ml of seed medium (glucose 2%, soluble starch 1%, soybean meal 2.5%, dried yeast 0.4%, meat extract 0.1%, NaCl 0.2%, K_2HPO_4

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0.005%, adjusted to pH 7.2). The fermentation was carried out on a rotary shaker (210 rpm) at 28°C for 48 hours. The culture was transferred to a 30-liter jar fermenter containing 18 liters of the same medium. The fermentation was carried out at 28°C with aeration of 10 liters/minute and agitation of 450 rpm for 24 hours. The seed culture was transferred to a 600-liter tank fermenter containing 400 liters of a modified medium (glucose 2%, soluble starch 3%, soybean meal 2.5%, dried yeast 0.4%, NaCl 0.2%, K_2HPO_4 0.005%, $CaCO_3$ 0.4%, adjusted to pH 7.2). The fermentation for production was carried out at 28°C with aeration of 200 liters/minute and agitation of 240 rpm for 114 hours.

Isolation and Purification

The whole broth (370 liters) was filtered and the mycelial cake (125 kg) was extracted with 90% acetone (400 liters). The acetone extract was concentrated *in vacuo*, and then extracted with ethyl acetate (200 liters). The ethyl acetate extract (450 g) was applied to a silica gel (1 kg) column chromatography (12 i.d. × 20 cm). After eluting with *n*-hexane (20 liters) and CH_2Cl_2 (2 liters), the column was eluted with MeOH - CH_2Cl_2 (1 : 99, 10 liters) to give an active fraction (14.8 g). This fraction was crystallized from MeOH and subsequently from MeCN to obtain crude staurosporine powder and a filtrate. The filtrate was evaporated to dryness and the residue (6.0 g) was applied to a Sephadex LH-20 column chromatography with methanol followed by reverse phase HPLC to yield 33 mg of RK-1409B. HPLC conditions were as follows; column: Capcell Pak type C_{18} (20 i.d. × 250 mm, Shiseido, Tokyo), solvent system: 80% MeOH containing 0.01% NH_4OH, flow rate: 5.0 ml/minute, UV detection at 290 nm.

Bleb Forming Assay and Inhibition of Protein Kinase C

The bleb forming assay utilizing K-562 human leukemia cells was described in previous papers[3,8]. Inhibition of protein kinase C was assayed by the bovine brain protein kinase C assay kit (Amersham).

Results and Discussion

Physico-chemical Properties

RK-1409B was obtained as a pale yellow powder, mp >260°C (dec), $[\alpha]_D^{22}$ +147° (*c* 0.2, DMSO). The molecular formula of RK-1409B was determined as $C_{27}H_{23}N_3O_4$ based on HREI-MS data (M^+ *m/z*: 453.1672, calcd: 453.1687). RK-1409B was soluble in DMSO and slightly soluble in MeOH, EtOAc, $CHCl_3$ and acetone, but insoluble in *n*-hexane and water. In the IR spectrum, absorption bands were observed at ν_{max}^{KBr} 3350, 2950, 1670, 1580, 1450, 1380, 1340, 1305, 1270, 1225, 1095 and 735 cm^{-1}. The UV spectrum is shown in Fig. 2; λ_{max}^{MeOH} nm (ε): 203 (37,870), 237 (sh, 24,460), 245 (25,370), 267 (sh, 26,270), 293 (58,710), 320 (sh, 10,420), 336 (13,140), 357 (9,970) and 374 (10,870).

Structural Elucidation

The UV spectrum of RK-1409B suggested the presence of an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5(6*H*)-one system as chromophore[9~12]. The molecular formula of RK-1409B, $C_{27}H_{23}N_3O_4$, was the same as that of RK-286C[12]. The UV and IR spectra of RK-1409B were indistinguishable from those of RK-286C. The ^1H NMR and ^{13}C NMR data of RK-1409B were summarized in Tables 1 and 2 and compared with those of RK-286C. Comparison of NMR data revealed the presence of the same indolocarbazole chromophore. Some differences were observed in the chemical shifts for sugar

Fig. 2. UV spectrum of RK-1409B (in MeOH).

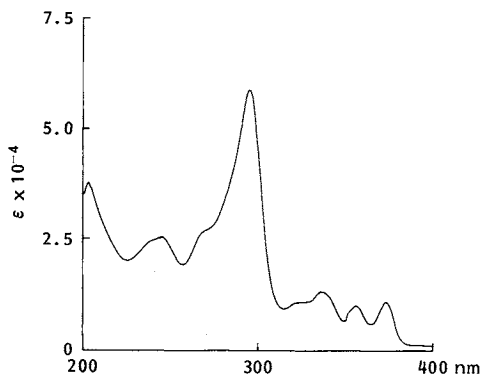


Table 1. ^{1}H NMR data of RK-1409B and RK-286C.

Protons	Chemical shifts[a] (J in Hz)		
	RK-1409B[b]	RK-1409B[c]	RK-286C[c,d]
1-H	7.38 (d, 7.9)	7.66 (d, 7.9)	7.59 (d, 8.1)
2-H	7.48 (dd, 7.9, 7.3)	7.47 (dd, 7.9, 7.3)	7.46 (dd, 8.1, 7.6)
3-H	7.33 (dd, 7.3, 7.3)	7.27 (dd, 7.9, 7.3)	7.28 (dd, 7.6, 7.6)
4-H	9.26 (d, 7.3)	9.24 (d, 7.9)	9.30 (d, 7.6)
6-H	—	8.51 (s)	8.45 (s)
7-H	4.91 (d, 17.1),	4.94 (d, 16.5),	4.89 (d, 16.8),
	4.97 (d, 17.1)	4.98 (d, 16.5)	4.97 (d, 16.8)
8-H	7.93 (d, 7.3)	8.06 (d, 7.9)	7.95 (d, 7.5)
9-H	7.36 (dd, 7.3, 7.3)	7.37 (dd, 7.9, 7.3)	7.27 (dd, 7.6, 7.5)
10-H	7.49 (dd, 7.3, 7.3)	7.53 (dd, 7.3, 7.3)	7.41 (dd, 7.6, 7.6)
11-H	7.69 (d, 7.3)	7.86 (d, 7.3)	7.99 (d, 7.6)
2′-Me	2.13 (s)	2.08 (s)	2.32 (s)
3′-H	4.09 (d, 5.5)	3.97 (d, 4.3)	3.84 (d, 3.8)
3′-OMe	3.74 (s)	3.69 (s)	3.42 (s)
4′-H	4.21 (ddd, 7.3, 5.5, 4.3)	4.07 (ddd, 5.5, 4.3, 4.3)	4.27 (m)
4′-OH	—	4.63 (br s)	4.17 (d, 3.6)
5′-H	2.21 (ddd, 14.0, 7.3, 4.9),	2.03 (ddd, 14.0, 5.5, 4.3),	2.14 (ddd, 15.0, 3.6, 1.0),
	2.71 (ddd, 14.0, 6.7, 4.3)	2.63 (ddd, 14.0, 5.5, 3.7)	2.61 (ddd, 15.0, 5.1, 3.2)
6′-H	6.65 (dd, 6.7, 4.9)	6.88 (dd, 6.1, 3.7)	6.77 (dd, 5.1, 1.0)

[a] Chemical shifts in ppm from TMS as an internal standard.
[b] $CDCl_3$-CD_3OD (1:9) as solvent.
[c] DMSO-d_6 as solvent.
[d] Data from ref 12.

Table 2. ^{13}C NMR data of RK-1409B and RK-286C.

Carbons	Chemical shifts[a]		Carbons	Chemical shifts[a]	
	RK-1409B[b]	RK-286C[c,d]		RK-1409B[b]	RK-286C[c,d]
C-1	107.4	108.5	C-10	124.7	124.1
C-2	125.3	124.7	C-11	112.2	115.7
C-3	119.7	118.9	C-11a	138.0	139.7
C-4	126.1	125.5	C-12a	129.3	129.5
C-4a	123.1	122.6	C-12b	125.7	126.2
C-4b	115.8	113.5	C-13a	136.8	136.1
C-4c	118.5	118.6	C-2′	94.0	90.9
C-5	173.8	172.2	C-3′	82.3	82.3
C-7	45.8	45.4	C-4′	65.1	58.8
C-7a	132.7	132.0	C-5′	32.4	29.0
C-7b	114.1	114.0	C-6′	80.0	79.5
C-7c	124.7	123.9	2′-Me	25.4	29.8
C-8	121.3	120.6	3′-OMe	59.8	56.5
C-9	120.3	119.6			

[a] Chemical shifts in ppm from TMS as an internal standard.
[b] $CDCl_3$-CD_3OD (1:9) as solvent.
[c] DMSO-d_6 as solvent.
[d] Data from ref 12.

moieties signals. In the ^{1}H NMR spectrum of RK-1409B, 3′-H, 3′-OMe and 6′-H signals were observed at lower field and 2′-Me, 4′-H and one of 5′-H signals were observed at upper field than those of RK-286C. These data indicated that RK-1409B is a stereoisomer of RK-286C. The ^{1}H and ^{13}C NMR assignments

Fig. 3. ^{1}H-^{13}C Long range coupling observed in HMBC spectrum of RK-1409B.

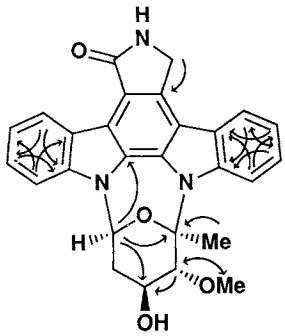
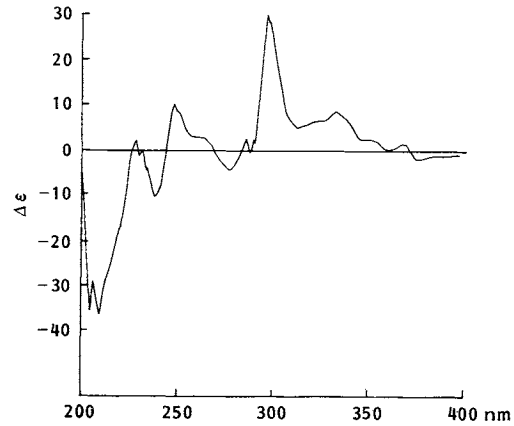


Fig. 4. CD spectrum of RK-1409B (in MeOH).



of RK-1409B were established by spin decoupling experiments and ^{1}H-^{13}C COSY. In HMBC experiments (Fig. 3), long range couplings from anomeric proton 6′-H (δ 6.65 ppm) to a hydroxymethine carbon C-4′ (δ 65.1 ppm) and two quaternary carbons C-12b (δ 125.7 ppm) and C-2′ (δ 94.0 ppm). Methyl group at δ 2.13 ppm was assigned to 2′-Me by long range coupling to C-2′. Observation of correlation peaks between methoxy group (δ_H 3.74 and δ_C 59.8 ppm) and oxygenated methine (δ_H 4.09 and δ_C 82.3 ppm) confirmed that the bonding position of methoxy group was C-3′. Connectivity of sugar moiety and aglycone was confirmed by NOE difference spectra. Irradiation of 11-H enhanced the intensity of 2′-Me and 3′-H. And NOEs between 1-H and 6′-H was also observed. These NOE data confirmed the attachment of C-2′ to N-12 and C-6′ to N-13. These data suggested that the planar structure of RK-1409B was the same as RK-286C.

Table 3. Inhibitory activities against bleb formation of K-562 cells and PKC *in vitro*.

	IC_{50} (μM)	
	Bleb suppression	PKC inhibition
RK-1409B	0.4	0.4
RK-286C	0.3	0.04
Staurosporine	0.002	0.002

Relative stereochemistry of the hydroxyl group at C-4′ was determined to axial by small coupling constants of 4′-H, $J_{3',4'}=4.3$ Hz, $J_{4',5a'}=5.5$ Hz and $J_{4',5b'}=4.3$ Hz. This evidence suggested that RK-1409B and RK-286C were epimers at C-3′ position. Axial orientation of the methoxy group at C-3′ was supported by observed NOE between 11-H and 3′-H. Absolute stereochemistry was determined by CD spectrum (Fig. 4). CD spectra of RK-1409B, RK-286C and staurosporine exhibited quite similar curves, and indicated that absolute stereochemistry at C-2′ and C-6′ were the same in those three compounds[12]. Using the above mentioned spectral data, the structure of RK-1409B was determined to 4′-demethylamino-4′-hydroxy-3′-epistaurosporine, which is a stereoisomer at C-3′ of RK-286C.

Biological Activity

Inhibitory activity of RK-1409B against protein kinase C and bleb formation induced by phorbol 12,13-dibutyrate (PDBu) were directly compared with that of RK-286C and staurosporine (Table 3). RK-1409B showed similar inhibitory activity as RK-286C on the bleb formation induced with PDBu. RK-1409B showed weaker inhibitory activity as compared to RK-286C with respect to *in vitro* PKC inhibition. In this paper, *in vitro* PKC activity was measured by PKC assay kit obtained from Amersham,

which contains bovine brain PKC and the synthetic peptide as the substrate. In previous papers[3,4], PKC from rabbit brain and type III histone were used. According to the difference of bioassay systems, IC_{50} value of RK-286C in this paper was different from that in a previous paper[4]. However, the order in activity is the same; staurosporine is the strongest among the three compounds. RK-1409B also inhibited the cell cycle progression at G_2 phase with polyploid DNA as same as RK-286C (data not shown). From the viewpoint of structure-activity relationships, the stereochemistry of the methoxy group at C-3′ is important and the equatorial orientation of the methoxy group is more effective than the axial orientation for inhibition of PKC.

RK-1409B showed weak antifungal activity similar to that of RK-286C. RK-1409B inhibited the growth of *Pyricularia oryzae* IFO 5994 at the concentration of 40 μg/disk, and no antimicrobial activity was observed among the other strains tested at this concentration (data not shown).

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