A NEW INHIBITOR OF PROTEIN KINASE C, RK-286C (4'-DEMETHYLAMINO-4'-HYDROXYSTAUROSPORINE)

II. ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE

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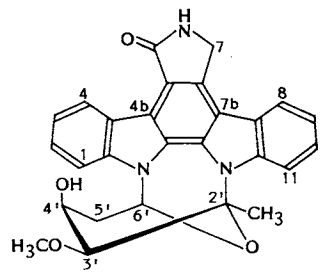
RK-286C, a new inhibitor of protein kinase C, has been found by the bleb-forming assay using K562 cells. It was produced by *Streptomyces* sp. RK-286 and purified by solvent extraction, silica gel chromatography and preparative HPLC. Spectrometric analysis revealed that the structure is 4'-demethylamino-4'-hydroxystaurosporine.

During screening using the bleb-forming assay[1], we identified staurosporine, isoflavones and sangivamycin[2] as inhibitors of protein kinase C (PKC). Recently, a new inhibitor of PKC was isolated from the culture filtrate and the mycelium extract of *Streptomyces* sp. RK-286. It is a new member of the indolocarbazole group of antibiotics, having the structure shown in Fig. 1. It showed inhibitory activities of PKC and platelet-aggregation *in vitro* as well as weak antifungal activity. Details of the fermentation and the biological activities were described in the preceding paper[3].

Fig. 1. Structure of RK-286C.

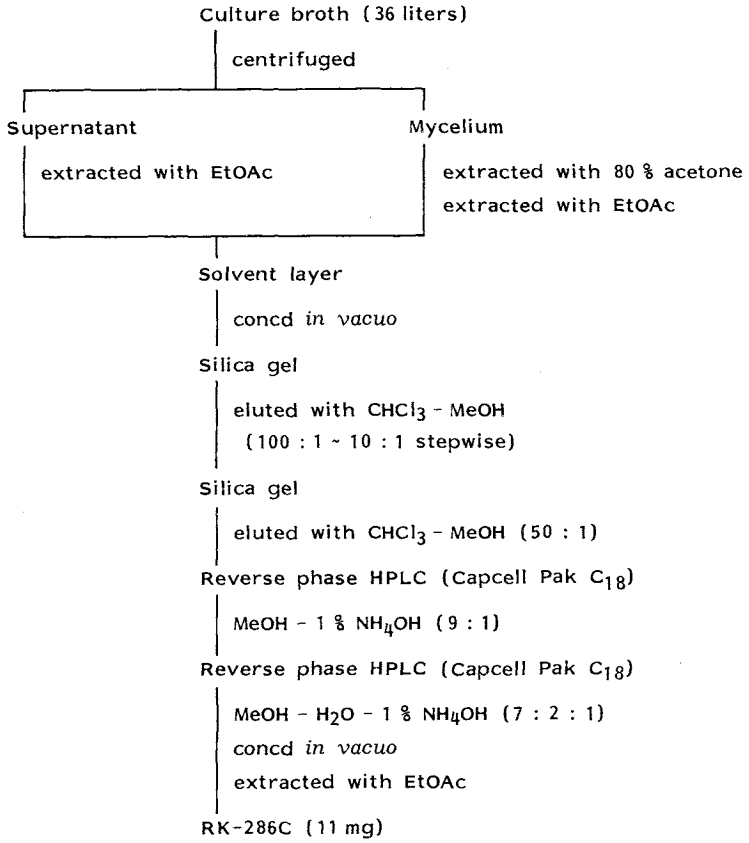


In this paper, we describe the isolation procedures, the physico-chemical properties and structure elucidation of RK-286C.

Isolation

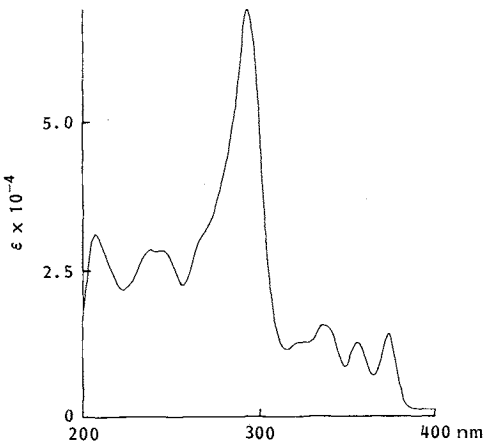
Fermentation was carried out as described in a previous paper[3]. As outlined in Fig. 2, culture broth (36 liters) was filtered and the mycelium was extracted with 2 liters of 80% acetone. The acetone extracts were concentrated *in vacuo*, then inhibitors were extracted with 1 liter of EtOAc. The active principle in the filtrate was also extracted with the same volume of EtOAc. The solvent layers were combined and concentrated *in vacuo* to dryness. The residue was applied onto a silica gel column and eluted stepwise by $CHCl_3$-MeOH (100:1 ~ 10:1). The active fractions eluted with $CHCl_3$-MeOH (10:1) were combined and concentrated *in vacuo* to dryness. The residue was applied onto a silica gel column again. It was developed with $CHCl_3$-MeOH (50:1) and the active fractions were collected. After concentration, inhibitors were separated by preparative HPLC. The HPLC was done twice by using a reverse phase column (Capcell Pak, 20 × 250 mm, monitored by UV at 292 nm). Solvent systems of the first and second preparative HPLC were MeOH-1% NH_4OH (9:1) and MeOH-H_2O-1% NH_4OH (7:2:1), respectively. By the first preparative HPLC, RK-286C was separated from staurosporine[4,5] (40 mg), and by the second

Fig. 2. Isolation procedure of RK-286C.



preparative HPLC, it was separated from K252c[6] (10 mg). RK-286C fractions were collected and evaporated to remove methanol and the aqueous solution was extracted with EtOAc. Solvent layer was evaporated *in vacuo* and lyophilized. Finally, 11 mg of pure RK-286C was obtained as a pale yellow powder. In the isolation process, fractions were monitored by both suppression of morphological change of K562 cells and HPLC analysis (UV profile and retention time).

Fig. 3. UV spectrum of RK-286C (in MeOH).

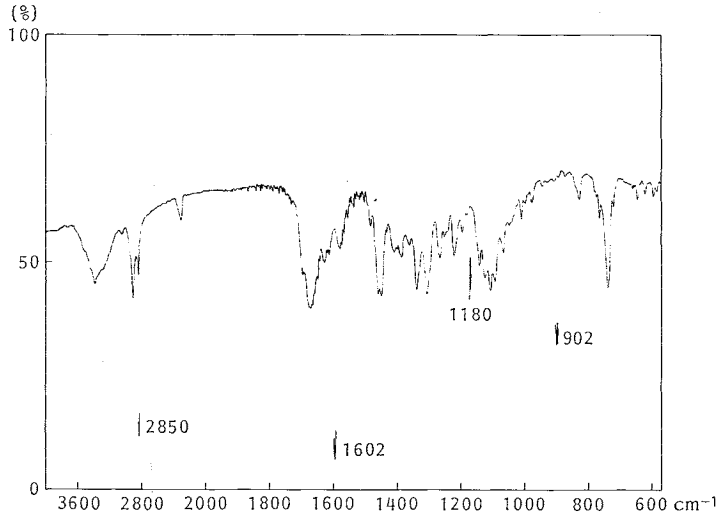


UV spectrum was measured on Hitachi 220A spectrophotometer.

Physico-chemical Properties

RK-286C was purified as a pale yellow powder, mp >265°C (dec), $[\alpha]_D^{20}$ +45.3° (*c* 0.22, EtOAc). The molecular formula of RK-286C was determined as $C_{27}H_{23}N_3O_4$ based on its HREI-MS data (M^+ *m/z* obsd: 453.1671, calcd: 453.1686). Mass spectrum was obtained on a Hitachi M-80. It is soluble in EtOAc and DMSO, slightly soluble in MeOH, but insoluble in water. The UV absorption spectrum was shown in Fig. 3; λ_{max}^{MeOH} nm (ε) 237 (30,450), 245 (30,120), 266 (sh, 31,770), 292 (69,500), 322 (sh, 14,230), 335

Fig. 4. IR spectrum of RK-286C (KBr).



IR spectrum was taken with Shimadzu IR27G recording IR spectrophotometer.

Table 1. ^{1}H NMR data of RK-286C and staurosporine with assignment (DMSO-d_6)[a].

Position	δ_H (ppm, J in Hz)	
	RK-286C	Staurosporine[b]
1	7.59 (d, 8.1)	7.56 (d, 8.1)
2	7.46 (ddd, 8.4, 7.6, 1)	7.45 (ddd, 8.1, 7.6, 1)
3	7.28 (d, 7.8)	7.27 (d, 7.6)
4	9.30 (d, 7.5)	9.30 (br d, 7.6)
6	8.45 (s)	8.51 (s)
7	4.89, 4.97 (d, 16.8)	4.95 (s)
8	7.95 (dd, 7.2, 1)	7.96 (dd, 7.1, 1.2)
9	7.27 (t, 7.5)	7.27 (t, 7.1)
10	7.41 (ddd, 8, 7.6, 1)	7.41 (ddd, 7.6, 7.1, 1.2)
11	7.99 (d, 7.6)	7.97 (d, 7.6)
3′	3.84 (d, 3.8)	4.03 (d, 3.7)
4′	4.27 (m)	3.24 (m)
$5'_{ax}$	2.41 (m, 14.9, 3.6, <1)	2.50 (m)
$5'_{eq}$	2.61 (m, 15.1, 6.0, 3.2)	
6′	6.77 (dd, 5.1, 1)	6.68 (dd, 4.9, 2.2)
2′-CH_3	2.32 (s)	2.29 (s)
3′-OCH_3	3.42 (s)	3.33 (s)
4′-NCH_3		1.44 (s)
4′-NH		0.76 (br)
4′-OH	4.17 (d, 3.6)	

[a] ^{1}H NMR spectrum was measured on JMN GSX-500 NMR spectrometer. TMS was used as an internal standard.
[b] Data from ref 6.

(16,550), 356 (13,240), 372 (14,890). No shift was observed under acidic and alkaline conditions. The IR spectrum (Fig. 4) indicated the presence of hydroxyl (3400 cm^{-1}), alkyl (3000~2850 cm^{-1}) and lactam (1680 cm^{-1}) groups.

Structural Analysis

The UV spectrum indicated that RK-286C has an indolocarbazole ring. The ^{1}H and ^{13}C NMR data are summarized in Tables 1 and 2 in comparison with those of staurosporine. The signals arising from

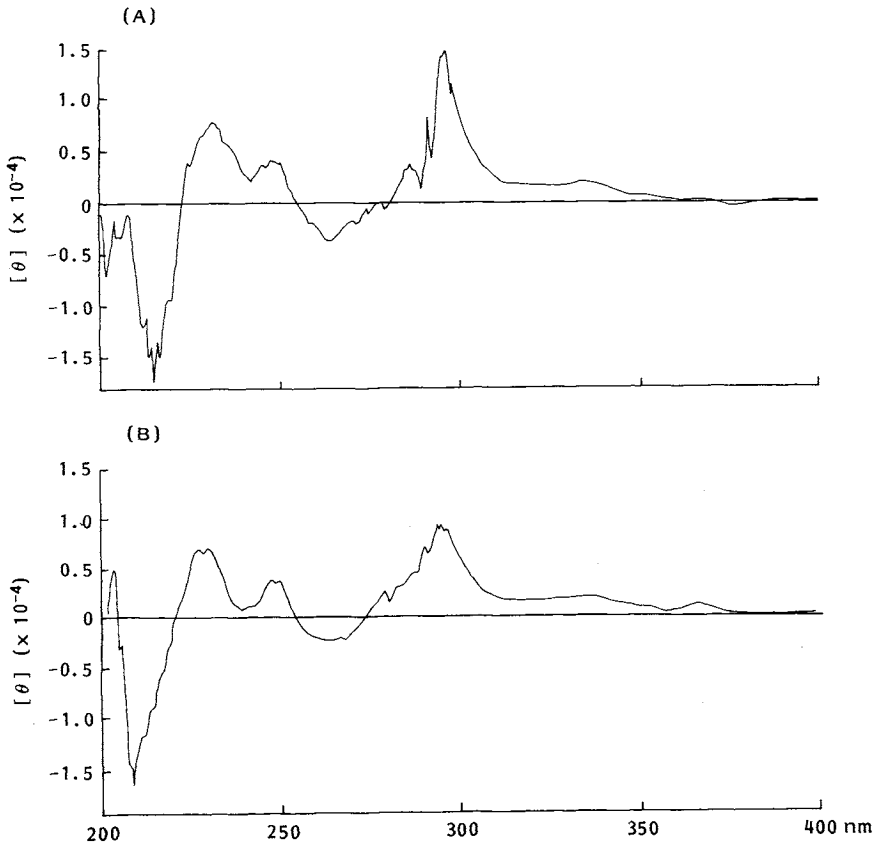
Table 2. ^{13}C NMR data of RK-286C and staurosporine with assignment (DMSO-d_6)[a].

Position	δ_c (ppm)		Position	δ_c (ppm)	
	RK-286C	Staurosporine[b]		RK-286C	Staurosporine[b]
1	108.5	108.2	10	124.1	124.2
2	124.7	124.8	11	115.7	115.1
3	118.9	118.9	11a	139.7	139.4
4	125.5	125.5	12a	129.5	129.9
4a	122.6	122.4	12b	126.2	126.6
4b	113.5	113.4	13a	136.1	136.3
4c	118.6	118.7	2′	90.9	91.0
5	172.2	172.2	3′	82.3	82.7
7	45.4	45.3	4′	58.8	50.0
7a	132.0	131.9	5′	29.0	29.3
7b	114.0	115.1	6′	79.5	79.8
7c	123.9	123.8	2′-CH_3	29.8	29.7
8	120.6	120.7	3′-OCH_3	56.5	57.2
9	119.6	119.6	4′-NCH_3		33.2

[a] ^{13}C NMR spectrum was measured on a Jeol FX-100FT spectrometer. TMS was used as an internal standard.
[b] Data from ref 6.

Fig. 5. CD spectra of RK-286C and staurosporine (in EtOH).

(A) RK-286C. (B) staurosporine.



CD curves were run on Jasco J-20A automatic recording spectropolarimeter.

the indolocarbazole moiety of RK-286C were assigned in comparison with ^{1}H and ^{13}C NMR data of staurosporine[7]. Concerning the sugar moiety, the signals of 3'-H, 5'-H, 6'-H, 2'-CH_3 and 3'-OCH_3 protons were observed at the same positions as those of staurosporine. The chemical shifts of C-2', C-3', C-5', C-6', 2'-CH_3 and 3'-OCH_3 were almost the same as those of staurosporine. On the other hand, considerable low field shifts of 4'-H (3.24→4.27 ppm) and C-4' (50.0→58.8 ppm) were observed. A proton signal at 4.17 ppm disappeared after addition of D_2O. In the ^{1}H-^{1}H COSY NMR, signals at 4.17 and 4.27 ppm were coupled to each other. The signals, corresponding to 4'-$NHCH_3$ of staurosporine, were not observed in ^{1}H and ^{13}C NMR spectra. These results suggested that a hydroxyl group is substituted in place of 4'-$NHCH_3$. The assignments of the sugar moiety was supported also by correlation in ^{13}C-^{1}H COSY NMR.

The stereochemistry of the sugar moiety was determined based on the coupling constants in comparison with those of staurosporine. The coupling constants ($J_{3',4'}=3.8$ Hz, $J_{5'_{ax},6'}=5.1$ Hz and $J_{5'_{eq},6'}=1$ Hz) suggested that 3'-H and 6'-H have the same configuration in the chair conformation as in the case of staurosporine. The J values between 4'-H and 5'-H ($J_{4',5'_{ax}}=3.2$ Hz and $J_{4',5'_{eq}}=3.6$ Hz) supported that 4'-H is equatorial. Therefore, the hydroxy group at C-4' is axial as in the case of 4'-$NHCH_3$ in staurosporine. Thus, the sugar moiety of RK-286C has relatively the same configuration and conformation as those of staurosporine. Similar CD curves of RK-286C and staurosporine indicate that the absolute configuration is the same in both compounds (Fig. 5). Therefore, we concluded that the structure of RK-286C is 4'-demethylamino-4'-hydroxystaurosporine.

Several PKC inhibitors and an antitumor antibiotic which have the indolocarbazole chromophore are reported[6,8,9]. RK-286C is a new member of this group having the structure, 4'-demethylamino-4'-hydroxystaurosporine.

Acknowledgments

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