A NEW INHIBITOR OF PROTEIN KINASE C, RK-1409 (7-OXOSTAUROSPORINE)

II. FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE

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RK-1409, a new inhibitor of protein kinase C, was isolated from the culture broth of *Streptomyces platensis* subsp. *malvinus* RK-1409. The structure was elucidated as 7-oxostaurosporine on the basis of spectroscopic analyses and oxidation of staurosporine.

In the course of screening using a bleb forming assay[1], we reported the novel inhibitors of protein kinase C (PKC) RK-286C (4'-demethylamino-4'-hydroxystaurosporine)[2] and RK-286D[3] previously. Recently, *Streptomyces platensis* subsp. *malvinus* RK-1409 was found to produce a new indolocarbazole group antibiotic, RK-1409 shown in Fig. 1. This compound inhibited PKC *in vitro* at an extremely low concentration.

In this paper, we describe the fermentation, isolation, physico-chemical properties, and structure elucidation. Details of the taxonomy and the biological activities were described in the preceding paper[4].

Experimental

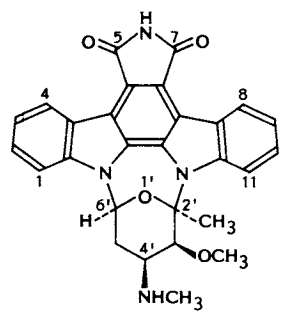
General

MP was measured with a Yanaco micro melting point apparatus. Optical rotation was measured by a Perkin-Elmer 241MC polarimeter. 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. ^{1}H NMR spectra were recorded on a Jeol GSX-500 spectrometer.

Fermentation

Streptomyces platensis subsp. *malvinus* RK-1409 was cultured in a 500-ml cylindrical flask containing 70 ml of seed medium (glucose 2%, soluble starch 1%, soybean meal 2.5%, meat extract 0.1%, dried yeast 0.4% and NaCl 0.2%, adjusted to pH 7.0). The fermentation was carried out on a rotary shaker (250 rpm) at 28°C for 72 hours. The seed 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 13 liters/minute and agitation of 350 rpm for 72 hours.

Fig. 1. Structure of RK-1409.



Isolation and Purification

Culture broth (18 liters) was filtered and the mycelium was extracted with 10 liters acetone. The

acetone extract was concentrated to 3 liters *in vacuo* and the aqueous residue extracted with equal volume of EtOAc three times. The EtOAc extract (8.3 g) was applied to a silica gel column with EtOAc-MeOH as eluent. The active fraction (548 mg) was eluted with EtOAc-MeOH (95:5) and chromatographed on silica gel columns with MeOH-$CHCl_3$ (5:95) and with MeCN-$CHCl_3$ (1:4). Further purification by Sephadex LH-20 column chromatography (MeOH) and reverse phase HPLC yielded RK-1409 (2.0 mg). Staurosporine[5] (160 mg) was also isolated from active fractions after the silica gel chromatography and recrystallization from MeOH.

HPLC Condition

HPLC analysis was performed using a Waters 600 Multisolvent Delivery System and Waters 990J Photodiode Array Detector. HPLC condition was as follows; column: Capcell Pak type C_{18} (4.6 i.d. $\times$ 250 mm, Shiseido, Tokyo) with precolumn (4.6 i.d. $\times$ 35 mm), solvent system: 80% MeOH containing 0.01% NH_4OH, flow rate: 1 ml/minute. Retention times of RK-1409 and staurosporine are 11.0 and 7.7 minutes, respectively.

Preparative HPLC was performed as follows; column: Capcell Pak type C_{18} (20 i.d. $\times$ 250 mm), solvent system: 80% MeOH containing 0.01% NH_4OH, flow rate: 5.0 ml/minute. Detection: UV at 315 nm. Retention time of RK-1409 was 31 minutes.

Oxidation of Staurosporine

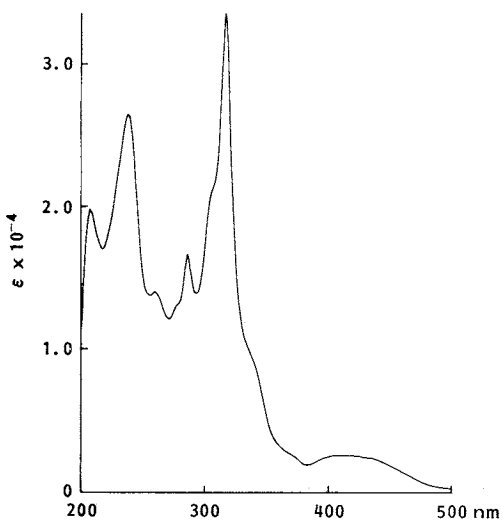
A solution of (+)-staurosporine (35 mg), 70% *tert*-butyl hydroperoxide (1 ml), Mn(III)acetylacetonate (2.64 mg) and EtOAc (1 ml) was stirred at 4°C. After stirring for 2 days Mn(III)acetylacetonate (2.64 mg) and EtOAc (0.5 ml) were added to the solution, and stirred further for 2 days at 4°C. The reaction mixture was subjected to silica gel (3.0 g) column chromatography eluted with EtOAc 15 ml and subsequently 10% MeOH-$CHCl_3$ 10 ml. The fraction of 10% MeOH-$CHCl_3$ was concentrated *in vacuo* and purified by HPLC. 7-Oxostaurosporine purified was 1.5 mg and staurosporine recovered was 5.0 mg. Synthetic 7-oxostaurosporine; MP >230°C (dec), $[\alpha]_D^{22}$ +36.9° (*c* 0.29, $CHCl_3$). Other spectral data and retention time on HPLC analysis were completely identical with natural RK-1409.

Results and Discussion

Physico-chemical Properties

RK-1409 was purified as a yellow powder, mp >235°C (dec), $[\alpha]_D^{20}$ +38.3° (*c* 0.06, $CHCl_3$). The molecular formula of RK-1409 was determined as $C_{28}H_{24}N_4O_4$ based on HREI-MS data (M^+ *m/z*: 480.1770, calcd: 480.1795). RK-1409 was soluble in MeOH, DMSO, EtOAc and $CHCl_3$ but insoluble in hexane and water. In the IR spectrum, absorption bands were observed at 3450, 2920, 1750, 1700, 1570, 1460, 1400, 1340, 1310 and 740 cm^{-1}. The UV spectrum is shown in Fig. 2; λ_{max}^{MeOH} nm (ε) 208 (19,600), 238 (26,400), 260 (13,600), 287 (16,800), 305 (sh, 20,800), 317 (33,600), 340 (sh, 8,800) and 410 (2,400).

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



Structure

The UV spectrum of RK-1409 suggested the presence of an indolo[2,3*a*]pyrrolo[3,4*c*]carbazole-5,7(6*H*)-dione system as chromophore[6,7]. The ^1H NMR data of RK-1409 and staurosporine are

Table 1. ^{1}H NMR data of RK-1409 and staurosporine ($CDCl_3$).

Protons	Chemical shifts[a] (J in Hz)		Protons	Chemical shifts[a] (J in Hz)	
	RK-1409	Staurosporine		RK-1409	Staurosporine
1-H	7.32 (br d, 7.6)	7.26 (d, 7.7)	11-H	7.91 (d, 8.5)	7.91 (d, 8.4)
2-H	7.57 (ddd, 7.6, 7.6, 1.2)	7.46 (t, 7.7)	2′-Me	2.37 (s)	2.33 (s)
			3′-H	3.88 (d, 3.7)	3.85 (d, 3.3)
3-H	7.43 (ddd, 7.9, 7.6, 1.2)	7.35 (t, 7.7)	3′-OMe	3.44 (s)	3.38 (s)
			4′-H	3.36 (ddd, 3.7, 3.7, 2.5)	3.32 (ddd, 3.3, 3.3, 3.1)
4-H	9.24 (br d, 7.9)	9.43 (d, 7.7)			
6-H	7.36 (br s)	6.55 (br s)	4′-NMe	1.56 (s)	1.54 (s)
7-H		5.03 (d, 15.5), 4.97 (d, 15.5)	5′-H	2.76 (br dd, 14.7, 2.5),	2.71 (dd, 14.7, 3.1),
				2.40 (ddd, 14.7, 5.5, 3.7)	2.37 (ddd, 14.7, 5.1, 3.3)
8-H	9.35 (d, 7.3)	7.87 (d, 7.7)			
9-H	7.37 (dd, 7.3, 7.3)	7.30 (t, 7.7)			
10-H	7.48 (dd, 8.5, 7.3)	7.40 (dd, 8.4, 7.7)	6′-H	6.53 (br d, 5.5)	6.53 (br d, 5.1)

[a] Chemical shifts in ppm from TMS as an internal standard.

summarized in Table 1. Comparison of the spectral data and decoupling experiments revealed the presence of two 1,2-disubstituted benzene rings and the same sugar moiety as that of staurosporine. The absence of the signal of the 7-H methylene protons and the chemical shift of a second deshielded aromatic proton at δ 9.35 ppm 8-H indicated the presence of a carbonyl group at C-7. These data suggested that the structure of RK-1409 was 7-oxostaurosporine. Oxidation of (+)-staurosporine with *tert*-butyl hydroperoxide and Mn(III)acetylacetonate[8] gave 7-oxostaurosporine. Spectral data of this oxidative product were identical with natural RK-1409. This oxidation confirmed the proposed structure of RK-1409, with the same absolute stereochemistry as that of staurosporine[9].

The structure of RK-1409 is a hybrid of a staurosporine type sugar moiety[9~11] and a 7-oxo-type indolocarbazole chromophore which is similar to the aglycone of the antitumor antibiotics rebeccamycin[6,12] and AT2433[7,13]. Therefore the biological activity of this novel antibiotic RK-1409 is interesting from the viewpoint of structure-activity relationships. Details of the inhibitory activities against some protein kinases were described in the preceding paper[4] and elsewhere.

After a preliminary report of these results[14] and the submission of this paper, we found that 7-oxostaurosporine was reported as an antitumor antibiotic BMY-41950 by Bristol-Myers Squibb Co. and an oxidative derivative of staurosporine by Kyowa Hakko Kogyo Co., Ltd. in patents[15,16].

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