CALPHOSTINS (UCN-1028), NOVEL AND SPECIFIC INHIBITORS OF PROTEIN KINASE C

I. FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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A novel complex of calphostin (UCN-1028), which specifically inhibits protein kinase C (PKC) has been isolated from the culture broth of a fungi *Cladosporium cladosporioides*. Purification of individual components was carried out by silica gel, non-porus resin Diaion HP-20SS and Sephadex LH-20 chromatography, leading to isolation of five closely related components, A, B, C, D and I. Calphostins showed cytotoxic activities against various tumor cells, and these cytotoxicities were proportional to their inhibitory activities against PKC.

Specific inhibitors of protein kinase C (PKC) can be excellent tools to study the role of PKC in cellular proliferation and differentiation, and are expected to be potential chemotherapeutic agents. In the course of screening new selective inhibitors of PKC, a fungi *Cladosporium cladosporioides* was found to produce a complex of novel and specific inhibitors of PKC, designated calphostin (originally called UCN-1028). The most hydrophobic component, calphostin A (UCN-1028A) which was the first purified compound, has already been reported[1]. Further study of the calphostin fermentation showed the presence of four components in addition to calphostin A.

This paper describes the fermentation, isolation, physico-chemical properties and biological activities of the calphostin complex.

Materials and Methods

Microorganism

The producing organism, *C. cladosporioides* FERM BP-1285 was isolated from a block fence around a house in Osaka, Japan.

Materials

Commercial reagents and their suppliers were: Histone type II-S and III-S, Sigma Chemical Co.; phosphatidylserine, Serdery Research Inc.; diolein, Nakarai Chemical Co.; [γ-^{32}P]adenosine triphosphate (ATP), Amersham; silica gel, Wako Pure Chemical Industries Ltd.; Diaion HP-20SS, Mitsubishi Chemical Industries Ltd.; Sephadex LH-20, Pharmacia; Nutrient Agar, Eiken Kizai Chemical Co.

Determination of Biological Activities

PKC was partially purified from rat brain by the method of KIKKAWA *et al.*[2] and PKC activity was assayed by measuring the incorporation of ^{32}P into histone. The reaction mixture (0.25 ml) contains Tris-HCl 20 mM (pH 7.5), magnesium acetate 10 mM, $CaCl_2$ 0.1 mM, phosphatidylserine 20 μg, diacylglycerol

0.8 μg, Histone type III-S 50 μg, partially purified enzyme 5 μg and [γ-^{32}P]ATP (10^5 cpm/nmol) 5 μM. Cyclic-AMP dependent protein kinase (PKA) was partially purified from bovine heart by the method of KUO and GREENGARD[3], and its activity was assayed as described by TAKAI *et al.*[4].

Human cervical cancer HeLa S_3 cells were cultured in modified EAGLE's minimum essential medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum (FBS). Human breast cancer MCF-7 cells were cultured in RPMI 1640 (GIBCO Laboratories) supplemented with 10% FBS, 10 μg/ml insulin, 10^{-8} M estradiol. For determination of cytotoxic activity of calphostin, the cells were preincubated for 24 hours at 37°C in 96-well plastic plates and then treated with different dilutions of drugs for 1 hour. After washing with PBS (−), these cells were cultured in fresh medium for 3 days. Thereafter the concentration of calphostin required for 50% inhibition of cell growth was determined by the uptake of neutral red dye by the modified method described previously[5].

Antimicrobial activity of calphostin was determined in nutrient agar by a 2-fold serial dilution method.

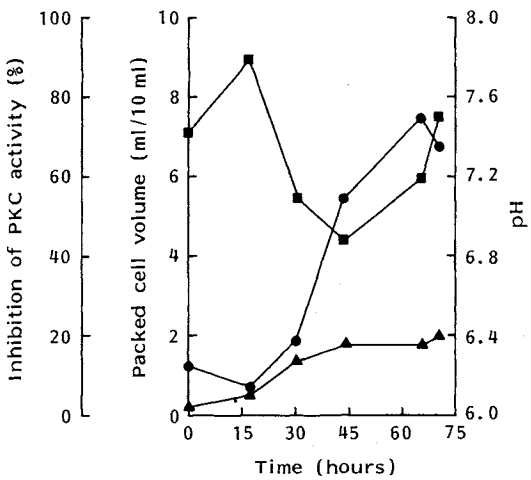
Results and Discussion

Fermentation

A loopful of well-sporulated stock culture was inoculated into the seed medium consisting of glucose 1%, Peptone (Kyokuto) 0.5%, dry yeast 0.5%, $CaCO_3$ 0.3% and V8 vegetable juice (Campbell) 20% (pH 6.0). After incubation at 25°C for 48 hours on a rotary shaker, 10 ml of the seed culture was inoculated into a 2-liter Erlenmeyer flask containing 300 ml of the same seed medium and incubated for 24 hours. The 1.8-liter of this second seed was transferred to a 30-liter jar fermenter containing 18 liters of the fermentation medium consisting of soluble starch 5%, dry yeast 1.5%, KH_2PO_4 0.05%, NaCl 2% $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.5% (pH 7.0). The fermentation was carried out at 25°C with an aeration of 1 vol/vol/minute and an agitation of 300 rpm. Mycelia of *C. cladosporioides* often attached to the wall of the jar fermenter during fermentation. This phenomenon was undesirable for efficient production of calphostin. Therefore, we examined the various fermentation conditions in order to prevent sticking. As a result, adding 2% sodium chloride was effective in preventing sticking. Under this fermentation condition microbial cell growth reached a plateau at about 40 hours after inoculation, and a maximum potency was achieved at 60 hours. A typical time course for the fermentation is shown in Fig. 1.

Fig. 1. Fermentation profile.

▲ Packed cell volume, ● inhibition of PKC activity, ■ pH.



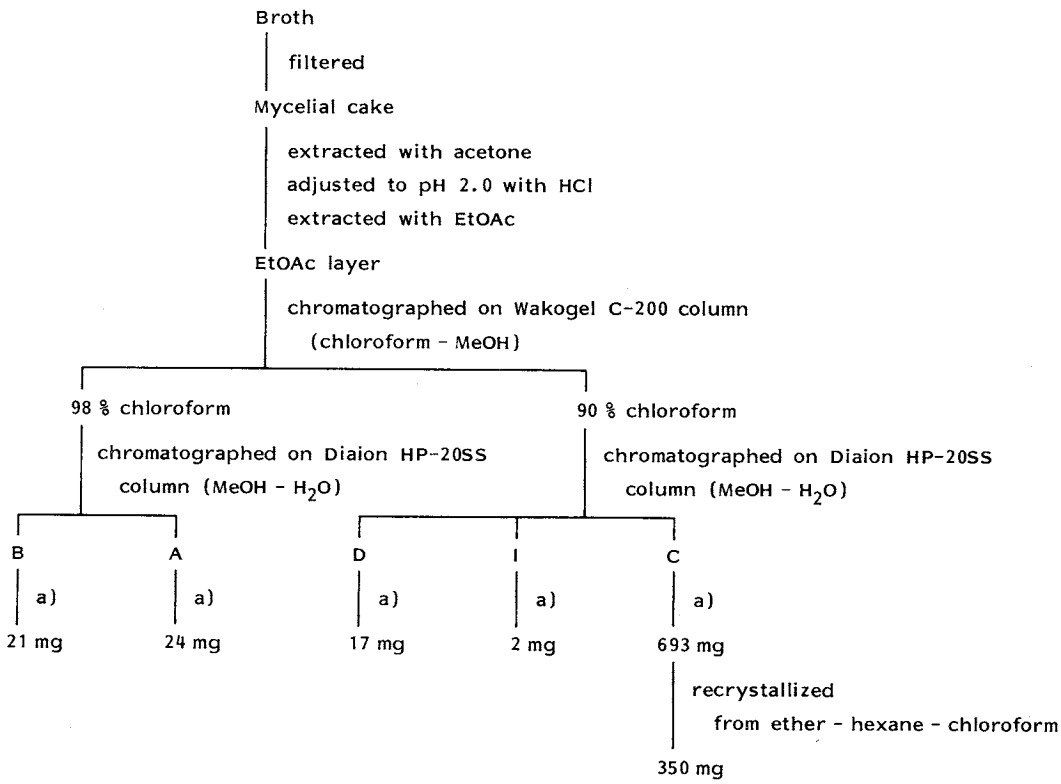
Microbial cell growth was determined by packed cell volume. 10 μl of the fermentation broth added to an equal volume of ethanol was used as assay sample. The definition of inhibition of PKC activity (%) is as follows;

$$1 - \frac{^{32}\text{P incorporation into histone in the presence of assay sample}}{^{32}\text{P incorporation into histone in the absence of assay sample}} \times 100$$

Isolation

Purification procedure for the isolation of each calphostin component is shown in Fig. 2. The mycelial cake from 30 liters of fermentation broth was extracted with acetone (10 liters × 3), because calphostin was accumulated in the mycelium. The

Fig. 2. Procedure for the isolation of calphostins.



a) Chromatographed on Sephadex LH-20 column; eluted with acetone.

combined acetone extract was concentrated *in vacuo* to aqueous solution, followed by extraction with ethyl acetate after adjustment pH 2.0 by hydrochloric acid. The organic solvent layer was dried over Na_2SO_4, and concentrated *in vacuo* to an oily mass, which was applied to a silica gel column (Wakogel C-200; 80 × 450 mm). The column was washed with *n*-hexane and chloroform, followed by elution with a mixture of chloroform - methanol (98 : 2) to yield calphostins A and B, and chloroform - methanol (9 : 1) to yield calphostins C, D and I. Each active fraction was pooled and concentrated *in vacuo* to an oily mass. The first fraction was applied to non-porus resin Diaion HP-20SS (40 × 600 mm) equilibrated with methanol - H_2O (1 : 1), and eluted stepwise with increasing concentration of methanol (90 and 95%) to yield calphostins B and A. The second fraction was also applied to Diaion HP-20SS column under the same condition as the first fraction, and eluted with 90, 95 and 100% methanol to yield calphostins D, I and C. Each component was further purified by Sephadex LH-20 (22 × 850 mm) chromatography equilibrated with acetone. Calphostins A (24 mg), B (21 mg), D (17 mg), I (2 mg) and C (693 mg) were obtained as shown in Fig. 2. Calphostin C, the major component, was recrystallized from ether - hexane - chloroform (9 : 4 : 1) to yield 350 mg of a dark red needle.

Physico-chemical Properties

Physico-chemical properties of each component are summarized in Table 1. They are readily soluble in dimethyl sulfoxide, chloroform, acetone and ethyl acetate, slightly soluble in methanol and insoluble in water and *n*-hexane. The UV spectra of the five components are similar and suggest the presence of

Table 1. Physico-chemical properties of calphostins.

	A	B	C	D	I
Nature	Dark red powder	Dark red powder	Dark red needle	Dark red powder	Dark red powder
MW (EI-MS, M^+)	758	654	790	550	806
Molecular formula	$C_{44}H_{38}O_{12}$	$C_{37}H_{34}O_{11}$	$C_{44}H_{38}O_{14}$	$C_{30}H_{30}O_{10}$	$C_{44}H_{38}O_{15}$
UV λ_{max}^{MeOH} nm	225, 269, 476, 543 (sh), 586	225, 269, 476, 543 (sh), 585	223, 270, 476, 543 (sh), 586	226, 269, 474, 539 (sh), 582	217, 258, 348, 475, 540 (sh), 582
TLC (Rf)[a]	0.21	0.36	0.32	0.54	0.44
HPLC (RT)[b]	7.2	3.2	4.4	1.6	—

EI: Electron impact.

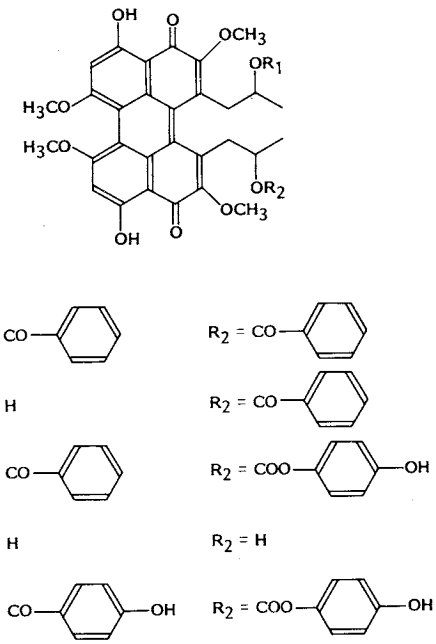
[a] ODS (Merck Art. No. 15685); H_2O-MeOH (1:4).

[b] RT: Retention time (minutes). Column: YMC-pack A-312 ODS, column size: 6×15 mm, mobile phase: 80% MeOH, flow rate: 2.5 ml/minute, detector: 585 nm.

Table 2. Inhibitory activity against PKC and PKA and *in vitro* cytotoxicity against HeLa S_3 and MCF-7 cells of calphostins.

	IC_{50} (μM)			
	PKC	PKA	HeLa S_3	MCF-7
A	0.25	>50	0.38	0.28
B	1.04	22.9	2.56	1.61
C	0.05	>50	0.23	0.18
D	6.36	12.7	8.45	2.69
I	0.14	>50	0.24	0.16

Fig. 3. Planar structures of calphostins.



perylenequinone[6]. Although these five components are very similar to each other, they can be distinguished by their chromatographic behaviors on TLC and HPLC systems. Planar structures of the individual components are shown in Fig. 3, and details of their structural determination are reported in the accompanying paper[7].

Biological Activities

As shown in Table 2 and Fig. 3, the inhibitory activity of each component against PKC is more potent with the increasing of aromatic moieties at C-14 and C-17 of calphostin. We have already clarified that the most potent calphostin C interacts with the regulatory domain of PKC[8]. Each component of calphostin showed cytotoxic activity against HeLa S_3 and MCF-7 cells (Table 2). Their cytotoxic activities are consistent with their inhibitory activities against PKC, suggesting that their cytotoxicities are due to the inhibition of PKC. In addition, the increase of aromatic moieties at C-14 and C-17 of calphostin contributes to the selective inhibition of PKC. Calphostins A, C and I which have aromatic moieties at both C-14 and C-17 inhibited PKC with an 50% inhibitory concentration (IC_{50}) 0.05~0.25 μM, but not PKA even at 50 μM. Therefore, aromatic moieties at C-14 and C-17 are significant for both potency and selectivity of inhibitory activity against PKC. It will be interesting

to determine whether the functional groups other than aromatic groups have similar effects on the inhibitory activity of calphostin.

Each component of UCN-1028 (100 μg/ml) was not active against the microorganisms tested except that only calphostin (UCN-1028C) showed weak activity against the Gram-positive bacteria *Streptococcus faecalis* (MIC 10.4 μg/ml).

The antitumor activity of calphostin is now being investigated in detail.

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