UCN-01 AND UCN-02, NEW SELECTIVE INHIBITORS OF PROTEIN KINASE C

I. SCREENING, PRODUCING ORGANISM AND FERMENTATION

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In the course of continued screening program for new selective inhibitors of protein kinase C (PKC), fermentation broths from over 5,000 soil isolates were screened for their inhibitory activity of PKC. HPLC analysis of active cultures revealed that five different strains (N-71, N-115, N-126, N-128 and N-139) of *Streptomyces* isolated from various local soil samples were found to produce staurosporine and related compounds. Of these strains, N-126, a high producing strain, was found to produce new selective inhibitors of PKC, UCN-01 and its stereoisomer, UCN-02. The pH control of fermentation resulted in an increase of the production of UCN-01 and UCN-02.

We have screened microorganisms isolated from soils and plants for their ability to produce new inhibitors of protein kinase C (PKC). As reported earlier, we have found: (a) Staurosporine inhibits protein kinases with IC_{50} values of 2.7 nM for PKC, 8.2 nM for protein kinase A (PKA) and 6.4 nM for tyrosine-specific protein kinase of p60 transforming protein of Rous sarcoma virus.[1,2] (b) A new selective inhibitor of PKC, UCN-01, has isolated and the planar structure of UCN-01 differs from staurosporine in that C-7 carbon bears a hydroxyl group. UCN-01 inhibits PKC with an IC_{50} value of 4.1 nM, while the IC_{50} value for PKA and $p60^{v\text{-}src}$ tyrosine specific protein kinase are 42 and 45 nM, respectively.[3]

In the course of our continued screening program for new selective inhibitors of PKC, several actinomycetes (strains N-71, N-115, N-126, N-128 and N-139) isolated from various local soil samples, were found to produce staurosporine[4,5] and also to produce staurosporine related compounds. Of these strains, N-126, a high producing strain, was used to study the improved production of new selective inhibitors, UCN-01 and co-production of minor component, UCN-02. This report describes the screening method, taxonomic comparison of producing organisms and fermentation with the strain N-126. Details of the isolation, physico-chemical properties and structure elucidation of UCN-01 and UCN-02 are reported in the following paper.[6]

Materials and Methods

Screening Methods

Strains of actinomycetes isolated from soil samples were inoculated into a 20-ml test tube containing 4 ml of sterile growth medium consisting of glucose 2.5%, corn steep liquor 1.5%, several vegitable protein 1.0%, cotton seed oil 0.5%, $CoCl_2 \cdot 6H_2O$ 0.001%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 7.0 prior to sterilization), and incubated at 28°C for 4 days on a rotary shaker. Culture broth was extracted with an equal volume of methanol. The extract (10 μl) was added to the reaction mixture of PKC

assay to determine their inhibitory activity.

Enzyme Assay

PKC was assayed in a reaction mixture (0.25 ml) containing 5 μmol of Tris-HCl, pH 7.5, 2.5 μmol of magnesium acetate, 50 μg of histone (Type III-S, Sigma Chemical Company), 20 μg of phosphatidyl serine, 0.88 μg of diolein, 125 nmol of $CaCl_2$, 1.25 nmol of [γ-^{32}P]ATP ($5\sim10\times10^4$ cpm/nmol) and 5 μg of partially purified enzyme from rat brain, according to the method of KIKKAWA *et al.*[7] PKA was partially purified from bovine heart according to the method of KUO and GREENGARD,[8] and similarly assayed as PKC assay except that 250 pmol of cAMP were added instead of phospholipid, diolein and $CaCl_2$. Histone (Type II-S, Sigma Chemical Company) and Tris-HCl, pH 6.8 were used.

Microorganisms

Strain N-126 was isolated from a soil collected in Okinawa Prefecture, Japan. Other strains were isolated from soil samples collected at different local sites, indicated in Table 1. Seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) and grown for 48 hours at 28°C. The seed medium consisted of glucose 10 g, soluble starch 10 g, yeast extract 5 g, Bacto-Tryptone 5 g, beef extract 3 g and $CaCO_3$ 2 g per liter of tap water. A 5%-vegetative seed was used to inoculate into the fermentation medium consisting of soluble starch 30 g, soy bean meal 5 g, KH_2PO_4 0.5 g, $MgSO_4\cdot 7H_2O$ 0.5 g and $CaCO_3$ 5 g per liter of tap water. The pH of media was adjusted to 7.0 prior to sterilization.

Characterization of Isolated Strains

Cell wall analyses were performed on cultures grown in SR-broth medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of tap water, pH 7.0) for 48 hours at 28°C. The mycelia were collected by centrifugation and were washed three times with distilled water. They were hydrolyzed in 6 N hydrochloric acid solution for 15 minutes at 121°C, and developed on a cellulose TLC with the solvent system, MeOH - H_2O - 1.0 N HCl - pyridine (160 : 35 : 5 : 20).

The methods and media recommended by the International Streptomyces Project (ISP)[9] for characterization of the *Streptomyces* species were employed. Color codes were assigned to the reverse pigments and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago).

The spores and mycelia of the strains were observed by scanning electron microscopy (scanning microscope model S-570 Hitachi Co., Ltd.).

HPLC Analysis

Whole fermentation broths were extracted with an equal volume of ethyl acetate. After centrifugation at $1,200\times g$ for 10 minutes, the ethyl acetate extracts were concentrated to dryness. The residues were reconstituted in methanol to a concentration 10 times that of the original fermentation broth for HPLC analysis. HPLC was performed using a Gasukuro-Kogyo Model 572 pump and a Union-giken MCPD-350PC stereomulti channel photo detector. A prepacked YMC-gel A-312 ODS (6×150 mm, 5 μm particle size, Yamamura Kagaku) was used with the solvent system 90% methanol containing 1% NH_4OH at a flow rate of 1 ml/minute (8 minutes run time). Retention times of UCN-01, UCN-02 and staurosporine detected by absorbance at 294 nm are 4.0, 3.3 and 4.6 minutes, respectively.

Results and Discussion

Screening of Selective Inhibitors of PKC

In the course of our screening program for new inhibitors of PKC, we have found that staurosporine inhibits PKC with IC_{50} value of 1 ng/ml. And so our screening system can detect even strains which produce staurosporine at concentration as low as a few ng/ml in the culture broth. We detected five strains whose broths showed more than 90% inhibition activity against PKC in the screening of 5,163 strains newly isolated during 1983 to 1985. By the following HPLC analysis, we found that

these all strains accumulated staurosporine and related compounds which have similar UV spectra. Thus, at least 0.1% of newly isolated actinomycetes were shown to produce staurosporine and the related compounds using a fixed culture condition. Of these strains, N-126, a high producing strain, was used to study the improved production of UCN-01 and other new products.

Comparison of Producing Strains

Strain N-126 produced well developed aerial mycelia, branched but not fragmented, with the sporophores hooked to loose coils of one to three turns. It was therefore placed in the *Retinaculum-Apertum* (RA) or *Spira* (S) section of the classification by PRIDHAM *et al.*[10] This morphology was readily observed on all media which supported the formation of aerial mycelia. The sporophores bore chains of 10 to 20 or occasionally more spores. Scanning electron micrographs indicated that the spore was cylindrical, from 1.1 to 2.0 μm by 0.5 to 0.4 μm in size, and with smooth surface. No sporangia, motile spore, vegetative mycelial spore, or sclerotium was observed (Fig. 1). Strain N-126 produced branched substrate mycelium which varied from cream to brownish color, depending on the medium. A brown water-soluble pigment was occasionally produced.

Various carbon sources were utilized (Table 1). Liquefaction of gelatin was negative, peptonization of milk was positive and the optimum growth temperature of the strain was around 30°C. A melanoid pigment was produced in tyrosine agar and peptone - yeast extract - iron agar medium. Analysis of the whole cell hydrolysate by TLC demonstrated the presence of LL-diaminopimeric acid. No *meso*-isomer was detected. This indicates that the cell wall belongs to cell wall type I.[11] The morphology and chemical characteristics of strain N-126 permitted a clear assignment of the organism to the genus *Streptomyces*. The strain N-126 was compared with *Streptomyces* species according to the methods described by SHIRLING and GOTTLIEB,[12~15] NONOMURA,[16] and WILLIAMS *et al.*,[17] and

Fig. 1. Scanning electron micrographs of strains N-71 (A), N-115 (B), N-126 (C), N-128 (D) and N-139 (E).

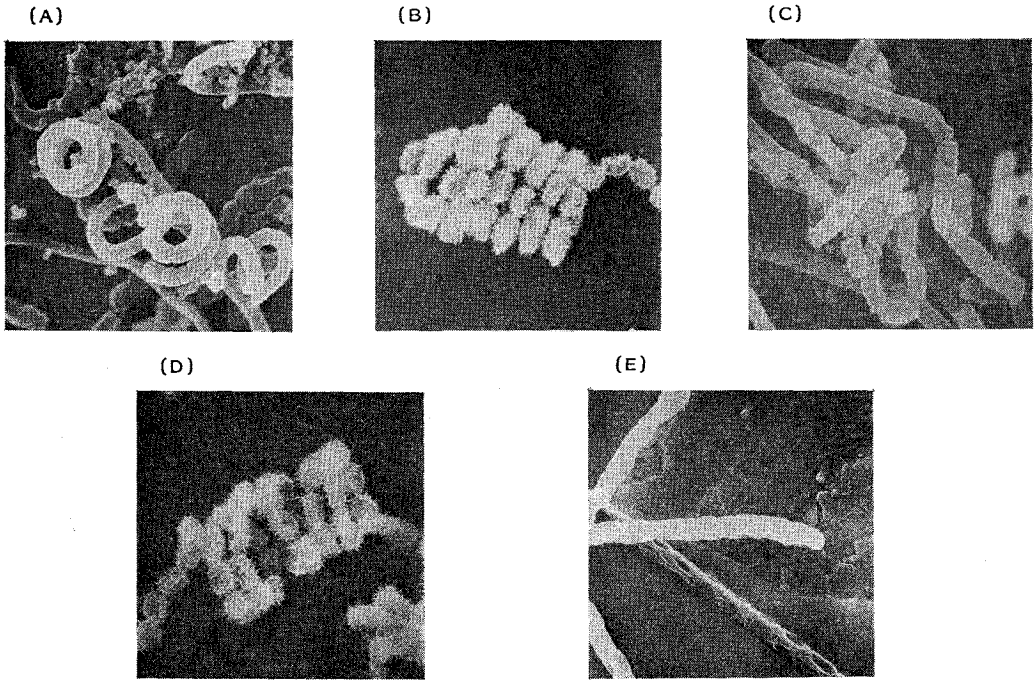


Table 1. Comparison of staurosporine producing strains.

	Strain				
	N-71	N-115	N-126	N-128	N-139
Aerial mass color	Gy	Pi, W	Gy, W	Pi, W	Gy, W, Y
Melanoid pigment	+	+	+	+	−
Soluble pigment	+	+	+	+	−
Spore chain	S	S	RA/S	RA/S	RF
Spore surface	sm	sp	sm	sp	sm
Liquefaction of gelatin	+	−	−	−	−
Peptonization of milk	+	+	+	+	+
Carbon utilization					
D-Glucose	+	+	+	+	+
L-Arabinose	−	+	+	+	+
D-Xylose	−	+	+	+	+
myo-Inositol	−	+	+	+	−
D-Mannitol	−	+	+	+	−
D-Fructose	−	+	+	+	−
L-Rhamnose	−	+	+	+	+
Sucrose	−	+	+	+	−
Raffinose	−	+	+	+	−
Salicin	−	−	+	+	+
D-Galactose	−	+	+	+	+
Species closely related	*S.*sp.	*S.c.*	*S.d.*	*S.c.*	*S.a.*
Collected place	Yamaguchi	Kyoto	Okinawa	Okinawa	Nagano

Gy; Gray, Pi; pink, W; white, Y; yellow, S; *Spira*, RA; *Retinaculum-Apertum*, RF; *Rectiflexibiles*, sm; smooth, sp; spiny.

*S.*sp.; *Streptomyces* sp., *S.c.*; *Streptomyces cyaneus*, *S.d.*; *Streptomyces diastaticus*, *S.a.*; *Streptomyces albidoflavus*.

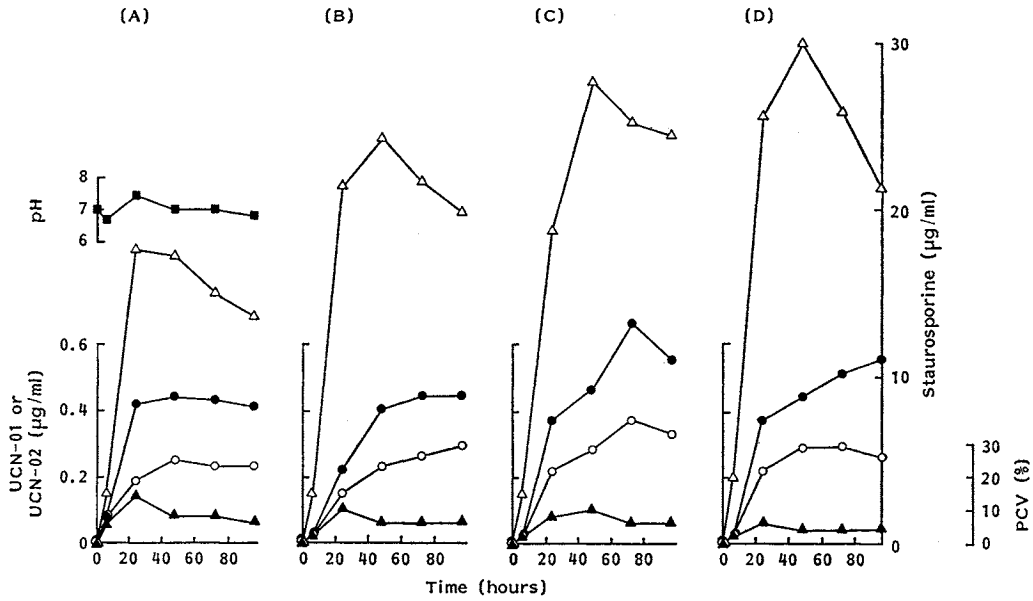
was closely resemble to *Streptomyces diastaticus* in WILLIAMS' classification.

In the same methods, the strains, N-71, N-115, N-128 and N-139, were found to belong to the genus of *Streptomyces*. The strain N-126 was compared with other strains as shown in Table 1. They are different from each other in the morphology and chemical characteristics, for example spore surface of strains N-115 and N-128 are spiny while that of strains N-71, N-126 and N-139 are smooth as shown in the scanning electron micrographs (Fig. 1). In the utility of various carbon sources, strain N-71 is not able to utilize any carbon sources except for glucose, while strains N-126 and N-128 can utilize various carbon sources. Staurosporine was first isolated from *Streptomyces staurosporeus*[4,5] and recently from *Streptomyces actuosus*[18] and now from above strains which are distributed among several different species of *Streptomyces*. Furthermore, structurally related metabolites such as K-252a,[19] SF-2370,[20] rebeccamycin[21] and AT2433[22] have been isolated from *Nocardiopsis* sp., *Actinomadura* sp., *Nocardia aerocolonigenes* and *Actinomadura melliaura*, respectively. These results indicate that the metabolites which have an indolo[2,3-*a*]carbazole system are produced by various strains of actinomycetes. Although it is well known that there are many protein kinases which play important role in cell growth of eukaryotes, those protein kinases of prokaryotes have not been reported. UCN-01, UCN-02, staurosporine and K-252a have potent inhibitory activities against protein kinases but have not shown the inhibitory activity against prokaryotes,[4,6,19] so that it is of great interest to determine whether actinomycetes produce such compounds in order to protect themselves from surrounding eukaryotes which have many significant protein kinases.

Fermentation

Fermentation titers of staurosporine, UCN-01 and UCN-02 using strain N-126 were increased by modifying the fermentation medium and the culture condition. We selected soluble starch and soybean meal as carbon and nitrogen sources of the fermentation medium with which the maximum yield of staurosporine increased about 3-fold comparing that of the previously described medium.[3] Fig. 2 shows the time course of fermentations using 5-liter jar fermentor. The peak titers of staurosporine, UCN-01 and UCN-02 reached after 24 hours of incubation under a condition without control of pH (Fig. 2A), the pH of the fermentation broth increased to around 7.5 after 24 hours and then gradually turned to around 7.0. The production of staurosporine reached a maximum of 18 μg/ml and the titers of UCN-01 and UCN-02 usually reached a maximum of 0.45 and 0.25 μg/ml, respectively, until 48 hours at 28°C. As described in the accompaning paper, UCN-02 is a stereoisomer of UCN-01 these stereoisomers are convertible each other in alkaline buffer solution above pH 7.0. And so, the effect of pH on titers of UCN-01 and UCN-02 was studied to learn whether one of the stereoisomers could be the biosynthetic product. In the pH control of fermentations (B~D), the production of staurosporine reached a maximum of 30 μg/ml with control at pH 5.8 (D). Under the conditions with pH control at 5.8 to 6.8, both UCN-01 and UCN-02 were accumulated as almost the same proportion as that seen without pH control. The highest production of UCN-01 and UCN-02 was achieved at pH 6.3 control (C) and maximum titers were 0.65 and 0.37 μg/ml, respectively, at 72 hours of cultivation. These results show that both UCN-01 and UCN-02 are able to be produced by strain N-126 under the condition in which it is hard for these compounds to be converted each other. The biosynthesis pathway of staurosporine, UCN-01 and UCN-02 remains to be investigated.

Fig. 2. The pH control of fermentations of strain N-126.
(A) pH no control, (B) pH 6.8, (C) pH 6.3, (D) pH 5.8.
■ pH, △ staurosporine, ● UCN-01, ○ UCN-02, ▲ packed cell volume (PCV).



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