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Isolation, and Biological Properties of a New Cell Cycle Inhibitor, Curvularol, Isolated from *Curvularia* sp. RK97-F166

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A new cell growth inhibitor, curvularol, was isolated from the fermentation broth of *Curvularia* sp. RK97-F166. Curvularol showed no antibacterial activity, and very weak antifungal activity. However, curvularol inhibited the cell cycle progression of normal rat kidney (NRK) cells in G_1 phase at 150 ng/ml. Curvularol induced the morphological reversion of src^{ts}-transformed NRK cells at 100 ng/ml, and inhibited protein synthesis same as cycloheximide.

It is known that products of oncogenes and tumor suppressor genes are involved in the mammalian cell cycle regulation[1,2]. Nowadays it becomes common understanding that disorder of the cell cycle regulation induces or enhances tumorigenesis. Low molecular weight compounds which normalize the cell cycle disturbance might be good candidates for antitumor agents. In the course of screening program for new microbial metabolites which inhibit the cell cycle progression, we have isolated a new inhibitor, curvularol, as an active principle from culture broth of *Curvularia* sp. RK97-F166. This paper describes taxinomy of the producing strain, and fermentation, isolation and biological properties of curvularol.

Materials and Methods

Taxonomic Studies

The producing microorganism, strain RK97-F166, was isolated from a leaf of an unidentified plant collected at Nobozaki, Nagasaki Pref., Japan. The strain was deposited at the National Institute of Bioscience and Human-Technology, Japan, under the accession number FERM P-17990. For species identification of strain RK97-F166, we referred to the description by ELLIS[3~5], SIVANESAN[6], ALCORN[7,8]. Colors of morphological structures and colonies were determined using the charts of KORNERUP and WANSCHER[9] (showing numeric-alphabetic-numeric codes in the form 26A2). For light microscopy, Nikon Biophot microscope was used with differential interference contrast (DIC) and a digital camera system (Kodak MDS 120).

In Vitro Antimicrobial and Cytotoxic Activities

In the *in vitro* antimicrobial and cytotoxic assays, curvularol was dissolved in dimethyl sulfoxide (DMSO) and diluted with each medium. The MIC's of curvularol were determined by the serial 2-fold dilution method in nutrient agar (composed of nutrient broth 1.8%, yeast extract 0.2%, and agar 1.3%) for bacteria, and in potato dextrose agar (composed of potato dextrose broth 2.4%, yeast extract 0.1%, and agar 1.3%) for yeasts and fungi.

Human erythroleukemia K562, human promonocytic leukemia U937, human promyelocytic leukemia HL60, rat pheochromocytoma PC12, human small cell lung carcinoma H1299, human epitheloid carcinoma HeLa, normal rat kidney NRK, and v-src transformed NRK cells, (1×10^4 cells/100 μl/well) were seeded on a 96-well

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microtiter plate (IWAKI glass) and incubated for 24 hours at 37°C in a CO_2 incubator. After the challenge with serially diluted curvularol for 48 hours, the cytotoxic effect was determined by modified MTT colorimetric method using WST-8 (Dojin Chemical Co.)[10]. The values of IC_{50} were estimated by graphic plots.

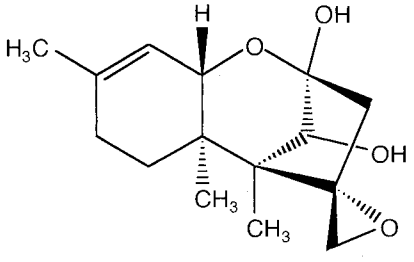
Analysis of Cell Cycle of *src*^{ts}-NRK Cells

NRK cells infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (*src*^{ts}-NRK)[11,12] were cultured at permissive temperature (32°C) or at restrictive temperature (39°C) in EAGLE's minimal essential medium (MEM) supplemented with 10% calf serum (CS, HyClone). The *src*^{ts}-NRK cells were plated at 1×10^5 cells/ml in a dish and incubated for 17 hours at 39°C for arresting the cell cycle at G_1 phase. The cells were simultaneously treated with various cencentrations of the curvularol, and transferred into 32°C to restore the cell cycle from G_1 arrest. After 17 hours, the cells were harvested and treated with PI staining solution (composed of 50 mg/ml propiodium iodide, sodium citrate 0.1%, and Nonident P-40 0.2%) at 4°C for 30 minutes. DNA histograms were obtained by using a flow cytometer (Beckman Coulter, EPICS Profile II) equipped with an argon-ion laser at 488 nm.

Analysis of Morphological Reversion of *src*^{ts}-NRK Cells

The *src*^{ts}-NRK cells maintained at 32°C were seeded into a 96-well microtiter plate at 2×10^4 cells/200 μl/well, and cultured for 4 hours at 32°C in a CO_2 incubator. The solutions of serially diluted curvularol (2 μl each) were added, and morphological reversion of *src*^{ts}-NRK cells was observed under a microscope after 17-hour incubation at 32°C. The activity was presented as the rate of normal flat cells in total cells. The value of ED_{50} was calculated by graphic plots.

Fig. 1. Structure of curvularol.



Macromolecule Synthesis

src^{ts}-NRK cells (1.5×10^5 cells/ml) were seeded onto a 48-multiwell plate in MEM supplemented with 10% CS and cultured at 32°C for 17 hours. The culture medium was changed to MEM containing 5% CS and [methyl-^{3}H]thymidine, [5,6-^{3}H]uridine, or [^{14}C-U]phenylalanine (finally 3.7 kBq/ml for ^{3}H and 0.37 kBq/ml for ^{14}C, Amersham Pharmacia) was added to the cell. The serial dilution of curvularol was added simultaneously. After 1-hour labeling, the cells were harvested and the acid-insoluble fractions were collected. The radioactivity was measured by a liquid scintillation counter.

Results and Discussion

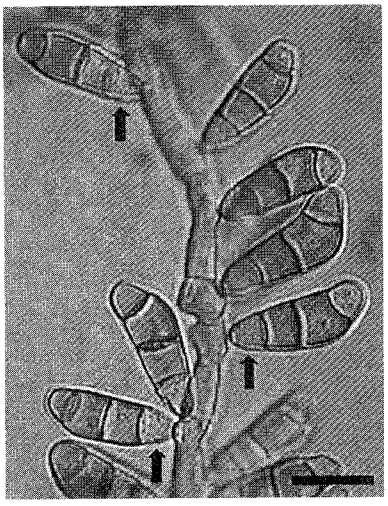
Taxonomy of Strain RK97-F166

Cultural and morphological characteristics of strain RK97-F166 described below with Fig. 2 suggested that the strain belonged to the hyphomycete genus *Curvularia*. Colonies on potato dextrose agar (PDA) were somewhat floccose, brownish grey (4D2-7D2), not zonate; reverse was almost black, and with abundant rhizomorph-like stromata visible. Mycelium on PDA was pale to dark brown, and composed of septate and branched hyphae, which were frequently nodular or swollen at the septum or intercalary. Stromata were produced on PDA in a few weeks, large, black to dark brown (7F8) on the surface, much paler on the inside, cylindrical, almost straight, irregularly bended or winded, and often branched irregularly. Conidiation was abundant on corn meal agar (CMA) and scanty on PDA. Conidiophores on CMA were arising terminally or laterally on the creeping hyphae, not on stromata, erect, not branched, often geniculate, septate, pale brown (6B2) to brown (7D8), smooth-walled, narrow at the base, thickening towards the apex, up to 400 μm long, and 4~6 μm thick in the broadest part. Conidiogenous cells were polytretic, integrated, terminal, intercalary, sympodially proliferated or regenerated, cylindrical, often swollen, and conspicuously darkly cicatrized. Tretoconidia on CMA were produced singly through conspicuous pores, straight or moderately to heavily curved between the third and fourth cells, ellipsoidal to clavate, with a darkly pigmented truncate base, 3-septate, almost smooth-walled, finely rough at the basal cell, and 14.5~22×5~10 μm (12~23×6~10 μm on PDA); the third cell from the base was usually larger and darker than the others; the cell at each end was usually pale brown (6B2); intermediate cells were brown (7D8).

Cultural and morphological features of the strain RK97-

Fig. 2. *Curvularia* sp. RK97-F166.

Slightly curved 3-septate tretoconidia, with the finely rough-surfaced basal cell (arrows), on CMA, DIC. Scale bar=10 μm.



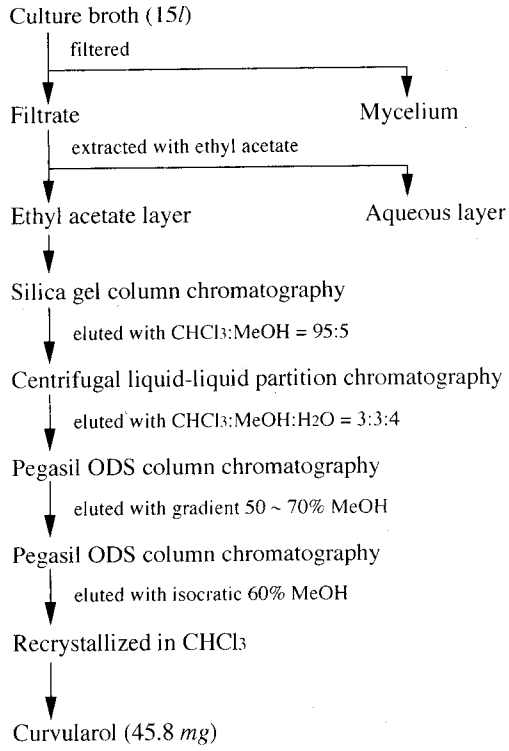
F166 are similar to those of *Curvularia lunata* var. *aeria* (BATISTA *et al.*) M. B. ELLIS, which produces stromata and 4-celled smooth conidia. The present strain, however, produced much smaller conidia than those of *C. lunata* var. *aeria*[3,6]. The basal cell of conidia in this strain is finely rough-surfaced on CMA (Fig. 2) and PDA, but not smooth as *C. lunata* var. *aeria*[3,6]. Therefore, we treat this strain as *Curvularia* sp. in this report.

Some derivatives of trichothecenes have been isolated from *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *etc.*[13], which are phylogenetically related to the Hypocreales ("Pyrenomycetes"). *Curvularia* and *Cochliobolus* teleomorph, however, belong to the Dothideales ("Loculoascomycetes").

Fermentation and Isolation

Strain 97-F166 was inoculated in ten 500-ml cylindrical flasks containing 70 ml of medium composed of glucose 1.5%, soluble starch 1.5%, malt extract 0.3%, dried yeast 1.0%, corn steep liquor 1.0%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.03% and agar 0.1%, adjusted to pH 6.0 before sterilization. After cultivation for 3 days at 28°C on a rotary shaker, this broth was transferred into a 30-liter fermentation tank containing 15 liters of the same medium, followed by cultivation for 48 hours at 28°C with agitation at 200 rpm, and aeration at 20 liters/minute.

Fig. 3. Isolation procedure of curvularol from *Curvularia* sp. RK97-F166.

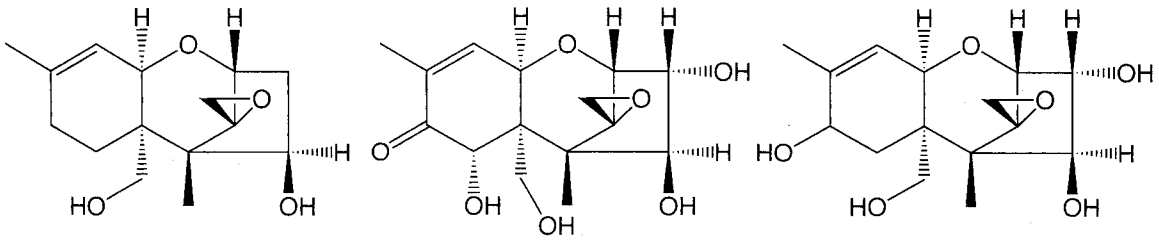


The fermentation broth of strain 97-F166 was filtered with filter papers (Whatman No. 2). The filtrate was extracted with the same volume of ethyl acetate. The ethyl acetate extract was concentrated, and then applied on a silica gel (Merck Silica gel-60) column which was prepared with $CHCl_3$. Active fractions, eluted with 95 : 5 in $CHCl_3$-MeOH, were collected, evaporated and applied on centrifugal liquid-liquid partition (Senshu Science Ltd., CPC-LLB-M) chromatography eluted with $CHCl_3$-MeOH-H_2O in 3 : 3 : 4. The active compound was purified on HPLC by ODS column eluted with gradient 50~70% MeOH, and further purified with isocratic 65% MeOH. After recrystallization in $CHCl_3$, an active principle, namely curvularol, was obtained as colorless needles. Isolation procedure of curvularol was summarized in Fig. 3. The structural determination and physico-chemical properties of curvularol are described in the separeted paper[14].

Antimicrobial and Cytotoxic Activity *In Vitro*

Biological activity of curvularol was compared with that

Fig. 4. Structures of verrucarol, nivalenol, and T-2 tetraol.



Trichothecene derivatives used in this study to compare with curvularol in biological activity.

Table 1. *In vitro* antimicrobial activity of curvularol and its related compounds.

Tested organisms	MIC (μg/ml)			
	Curvularol	Verrucarol	Nivalenol	T-2 tetraol
Escherichia coli JCM1649	>100	>50	>50	>50
Pseudomonas aeruginosa JCM2776	>100	50	>50	>50
Xanthomonas citri IFO3781	>100	-[a]	-	-
Bacillus subtilius IFO3513	>100	>50	>50	>50
Staphylococcus aureus IFO12732	>100	>50	>50	>50
Saccharomyces cerevisiae JCM2215	100	>50	>50	>50
Schizosaccharomyces pombe JCM1846	25	>50	>50	>50
Candida albicans JCM1542	>100	>50	>50	>50
Aspergillius niger JCM5697	>100	>50	>50	>50
Fusarium oxysporum JCM9284	>100	-	-	-
Penicillium chrysogenum JCM2056	>100	>50	>50	>50
Pyricuralia oryzae IFO5994	100	>50	>50	>50
Rhizopus oryzae JCM5559	>100	-	-	-

Bacterial strains were cultured at 37°C, and eukaryotic microorganisms were cultured at 28°C.
a: Not tested.

of other related compounds (Fig. 4). Curvularol did not show any inhibitory activity against Gram-negative and Gram-positive bacteria up to 100 μg/ml. Curvularol exhibited very weak antifungal activity to *Saccharomyces cerevisiae* JCM2215, *Schizosaccharomyces pombe* JCM1846, and *Pyricularia oryzae* IFO5994 (Table 1).

However, curvularol strongly inhibited growth of K562, U937, HL60, PC12, and H1299 cells: the range of IC_{50} values was 40~90 ng/ml. The growth of HeLa, NRK, and *src*^{ts}-NRK were inhibited at 430, 150, and 100 ng/ml, respectively (Table 2).

Cell Cycle Inhibition and Morphological Reversion on *src*^{ts}-NRK Cells

When *src*^{ts}-NRK cells were cultured at 32°C, spherical transformed cells were observed. Then cultured at 39°C, flat normal cells appeared instead of spherical cells. Curvularol at 30 ng/ml induced morphological reversion of *src*^{ts}-NRK cells from spherical transformed morphology to flat normal morphology at 32°C (Fig. 5). As shown in Table 2, IC_{50} value of curvularol for *src*^{ts}-NRK cells was 100 ng/ml. However, curvularol exhibited not cytocidal but cytostatic effect at the concentration up to about 10 μg/ml.

Table 2. *In vitro* cytotoxic activity of curvularol and its related compounds.

Tested cell lines	IC_{50} (ng/ml)			
	Curvularol	Verrucarol	Nivalenol	T-2 tetraol
K562	55	2200	80	170
U937	40	-[a]	-	-
HL60	40	-	-	-
PC12	90	-	-	-
H1299	75	-	-	-
HeLa	430	-	-	-
NRK	150	12000	6300	3000
src^{ts}-NRK	100	8500	300	800

All cells were cultured at 37°C except *src*^{ts}-NRK cells, which were cultured at 32°C.
a: Not tested.

Fig. 5. Morphological change of *src*^{ts}-NRK cells.

Cells were cultured at 39°C (restrictive temperature) (A), at 32°C (permissive temperature) (B). After addition of curvularol to give 100 ng/ml and cultivation at 32°C for 17 hours, morphologically reversed cells were observed (C).

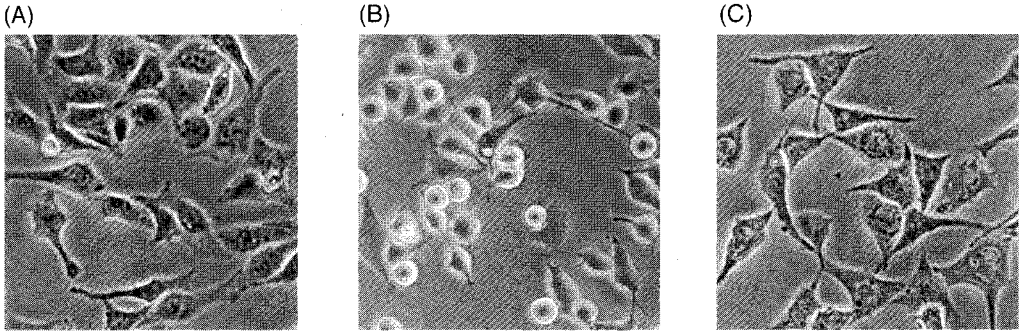


Fig. 6. Cell cycle analysis of *src*^{ts}-NRK cells treated with curvlarol.

Cells were cultured at 32°C (permissive temperature) (A), at 39°C (restrictive temperature) for 17 hours (B). After addition of curvularol to give 50 ng/ml and cultivation at 32°C for 17 hours, DNA contents were analyzed by flow cytometer (C).

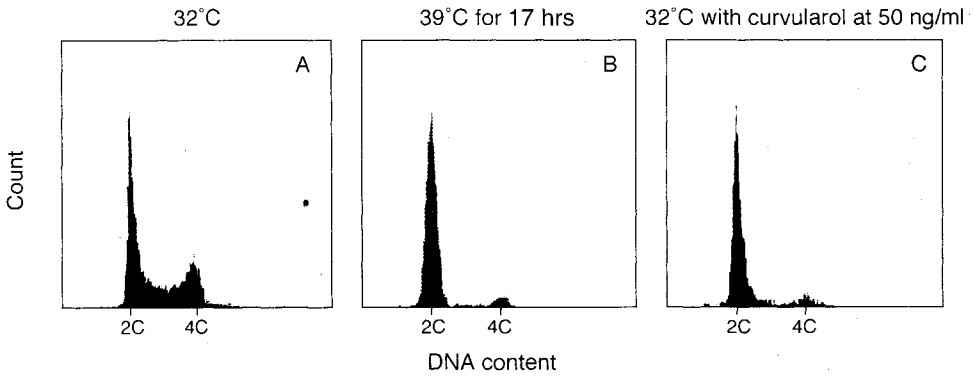
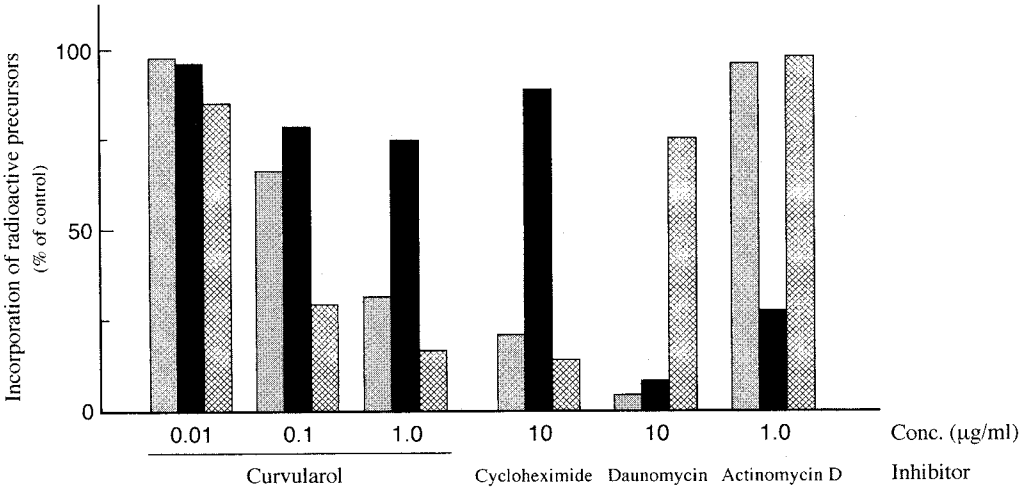


Fig. 7. Incorporation of radioactive precursors into acid-insoluble fraction of *src*^{ts}-NRK cells.



Radioactivity was presented in percentages of scintillation counts compared with control. Radioactive precursors were used as follows: dotted bars: [methyl-^{3}H]thymidine, solid bars: [5,6-^{3}H]uridine, and meshed bars: [^{14}C-U]phenylalanine.

Flow cytometric analysis revealed that curvularol inhibited the cell cycle progression of *src*^{ts}-NRK cells at G_1 phase (Fig. 6).

Effect of Curvularol on Macromolecular Synthesis

Curvularol inhibited protein synthesis stronger than DNA or RNA synthesis as shown in Fig. 7. It was reported that eukaryotic protein synthesis inhibitors, such as cycloheximide[15], reveromycin A[16], modulated the morphological reversion of *src*^{ts}-NRK cells. Curvularol primarily affected protein synthesis more than DNA and RNA syntheses. Curvularol is structurally related to the trichothecenes, however curvularol possesses unique structure different from other trichothecenes, such as trichodermol, verrucarol and T-2 toxins[17].

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