FUNICULOSIN, A NEW ANTIBIOTIC. I
ISOLATION, BIOLOGICAL AND CHEMICAL PROPERTIES

(Studies on Antiviral and Antitumor Antibiotics. XIII)

KUNIO ANDO, SEIKICHI SUZUKI*, TETSUJI SAEKI,
GAKUZO TAMURA and KEI ARIMA

Laboratory of Microbiology, Department of Agricultural Chemistry,
the University of Tokyo, Tokyo, Japan
* Research Laboratories, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan

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A new antibiotic, funiculosin, $C_{27}H_{41}NO_7$, was isolated from the filter cake of the fermented broth of *Penicillium funiculosum* THOM. Funiculosin is a neutral lipophilic substance which inhibits both DNA and RNA viruses as tested in infected primary chick embryo fibroblast cell monolayer and also some pathogenic fungi such as *Tricophyton mentagrophytes* and *Candida albicans*.

In our screening for antiviral and antitumor antibiotics, a new antibiotic, funiculosin, $C_{27}H_{41}NO_7$, was isolated in crystalline form from the filter cake of the culture medium of *Penicillium funiculosum* THOM IAM 7013, a type culture preserved in our laboratory. According to THOM's description, the fungus is ubiqiutous and has world-wide distribution[1]. It had been reported that *P. funiculosum* produced an antiviral antibiotic, helenine, found in the mycelium[2]. Funiculosin, however, is clearly different from helenine in its biological and chemical properties. Funiculosin has an antifungal activity at low concentrations. It is substance with a low molecular weight. In this paper we summarize the isolation, the biological and some chemical properties of funiculosin.

Production and Isolation

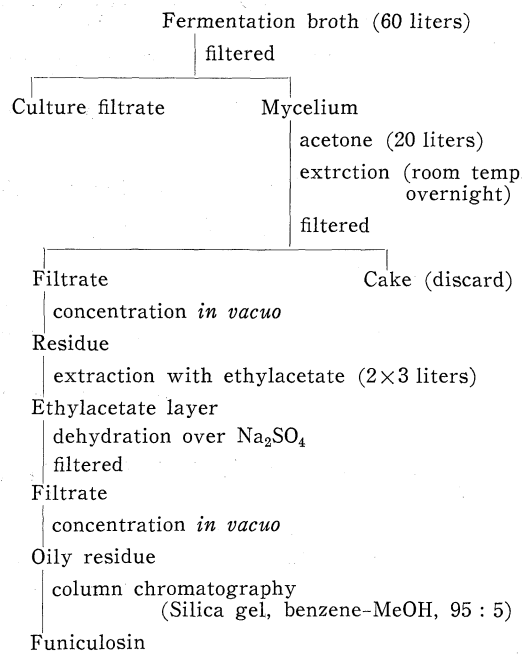
In a primary screening for antiviral antibiotics using HERRMAN's paper-disc agar-diffusion plaque-inhibition method[3], an acetone extract of the filter cake of the fermented broth of *Penicillium funiculosum* THOM IAM 7013 showed antiviral activity against Newcastle disease virus strain Miyadera growing on primary chick embryo fibroblast monolayer (CEF). This activity was noted when the fungus was cultured in a medium composed of glucose, lactose and corn steep liquor as chief nutrients.

The metablic products of the genus *Penicillium* have been extensively studied and 2, 6-dipicolinic acid[4], spiculisporic acid[5], miniloluteic acid[6] and islandicin[7] have been isolated from *P. funiculosum*. Our mycelial extracts produced a growth inhibitory activity with *Candida albicans* strain Ch, and we observed that the extract inhibited surface growth of the organism without inhibiting the inner growth. Thus, when the antimicrobial spectrum was determined by the paper-discs impregnated with the extract, clear growth inhibitory zones were formed against filamentous fungi such as

Tricophyton mentagrophytes in contrast to the turbid zone against *C. albicans*.

The hypothesis that the antiviral antibiotic would be responsible for antifungal activity was supported by the bioautograms using silica gel thin-layer chromatography, in which both antiviral and antifungal activities showed the same Rf values in various solvent systems. An agar-diffusion assay using *C. albicans* as test organism was developed and used in fractionation and isolation of the active material.

Fig. 1. Extraction and isolation



The procedures for extraction and fractionation are summarized in Fig. 1. *P. funiculosum* was grown aerobically for 4 days at 27°C in a stirred jar fermentor. The pulpy mycelium harvested was washed thoroughly with water and then extracted with acetone. The procedures for extraction, fractionation and isolation were essentially the same as previously reported[8]. The active principle was obtained as colorless needles from the crude mycelial extract by silica gel column chromatography followed by concentration of the combined active fractions. Our antibiotic was named funiculosin, because it is a specific metabolic product of *P. funiculosum*.

Biological Properties

1. Antiviral activity *in vitro*: Funiculosin inhibits plaque formation by Newcastle disease virus (NDV) and herpes simplex virus strain HF in infected chick embryo fibroblast (CEF). As shown in Table 1, the minimal inhibitory doses against these viruses are 20 mcg/ml and the minimal cytotoxic dose to CEF is 160 mcg/ml. The chemotherapeutic index is not so high in this assay system as compared with those of mycophenolic acid[9] and brefeldin A[10].

When HeLa cell monolayer was grown in tubes in the presence of funiculosin, cytotoxicity was observed at a concentration of 1 mcg/ml along with drop in pH of the medium in comparison with control. The drop apparantly occurred due to stimulation of high rate of glycolysis and accumulation of lactic acid in the medium. In this respect funiculosin might belong to the same class of antibiotics as nonactin, ossamycin[11] and ascochlorin[8].

At lower concentrations than 1 mcg/ml, the antibiotic was unable to suppress the cytopathic effect (CPE) of HeLa cells infected with NDV, herpes simplex virus and vaccinia virus strain DIE. Although funiculosin is effective against some viruses in the agar-diffusion method, it is unlikely that the antibiotic exerts prophylactic activity *in vivo* against virus infections.

2. Antitumor activity: It was considered that antitumor activity would be closely related to antiviral activity, since many antitumor agents show antiviral activity either *in vitro* or *in vivo* under certain conditions. The antitumor activity of funiculosin was determined by EHRLICH ascites tumor-mouse system. Mice (strain *ddY*, 5 weeks old) weighing 18~22 g were implanted intraperitoneally with 2×10^6 cells of EHRLICH ascites tumor. The treatment was initiated 24 hours after implantation by injecting intraperitoneally funiculosin solution in 0.2 ml of phosphate buffered saline (pH 7.0), once daily for 6 consecutive days. The effect was determined by body weight gain caused by tumor growth and survival times of the treated mice comparing with those of the untreated control.

As shown in Fig. 2, funiculosin is slightly effective against the tumor because inhibition of the tumor growth and prolongation of life span were observed at doses of 20 and 5 mcg/mouse/day. However, the high acute toxicity, LD_{50} 4 mg/kg in mice by intraperitoneal injection, precluded testing higher doses.

3. Antimicrobial activity: The antimicrobial spectrum of funiculosin is shown in Table 2. The antibiotic is a broad spectrum antifungal antibiotic which inhibits the growth of some pathogenic fungi. The inhibitory activity against *Trichophyton mentagrophytes* and *Piricularia oryzae* is especially noteworthy: the growth of the former is inhibited at a concentration of 2 mcg/ml and that of the latter at below 1 mcg/ml. But the antibiotic is ineffective against both gram-positive and negative bacteria even at 100 mcg/ml. In spite of its high acute toxicity,

Fig. 2. Antitumor activity of funiculosin

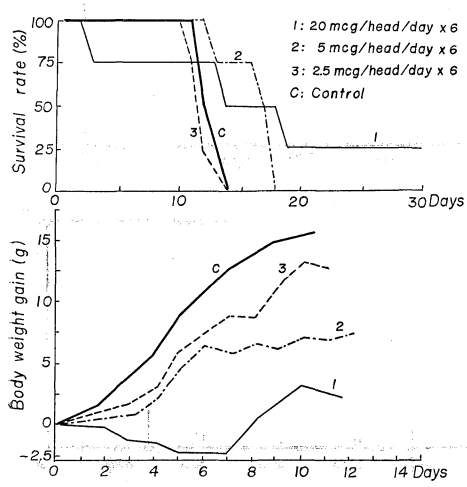


Table 1. Antiviral activity of funiculosin in the agar-diffusion plaque-inhibition method

Concentration (mcg/ml)	Antiviral activty (mm)	
	CZ	IZ
1,280	14	27
640	13	25
320	12	22
160	10	21
80	—	17
40	—	15
20	—	10
10	—	—

Newcastle disease virus strain Miyadera (NDV) and chick embryo fibroblast monolayer cell-host system was used. Antiviral activity is expressed as diameter of plaque-free protected zone (IZ) together with inner cytotoxic zone (CZ). The paper-disc used was of 8 mm in diameter and 1 mm in thickness whose absorbing capacity is 0.05 ml/disc.

Table 2. Antimicrobial spectrum of funiculosin

Organisms	Minimal inhibitory concentration (mcg/ml)
Candida albicans	2
Candida utilis	5
Cryptococcus neoformans	2
Saccharomyces cerevisiae	20
Aspergillus niger	40
Piricularia oryzae	<1
Trycophyton mentagrophytes	2
Trycophyton astroides	5
Bacillus subtilis	>100
Escherichia coli	>100

funiculosin is not inflammatory to guinea pig skin when applied as an alcoholic solution.

Chemical Properties

Funiculosin is a colorless lipophilic substance that is readily recrystallized from ethylacetate as needle-shaped crystals. It is soluble in pyridine, acetone and alcohol; sparingly soluble in ethylacetate, diethyl ether, dioxane, benzene and chloroform; insoluble in hexane and water. The antibiotic shows no definite melting point; the crystalline material began to turn yellow at 120°C and decomposes at 154~155°C

The molecular formula, $C_{27}H_{41}NO_7$, was assigned to funiculosin on the basis of the molecular weight, 491, determined by mass spectroscope and microelementary analyses.

Reactions with ferric chloride and TOLLENS reagent were negative so that neither phenolic nor enolic hydroxyl nor aldehyde groupings were present in the molecule. Negative ELSON-MORGAN and ninhydrin reactions indicated that aminosugar moiety was not involved in the molecule. The presence of olefinic bond suggested by immediate decoloration with $KMnO_4$ in acetone solution, which was supported by an ultraviolet molar absorptivity, 5,500 at 290 mμ in methanol.

Fig. 3. Infrared spectrum (nujol)

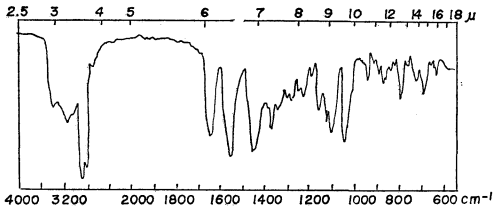


Fig. 4. NMR spectrum of acetylfuniculosin (solvent : $CDCl_3$)

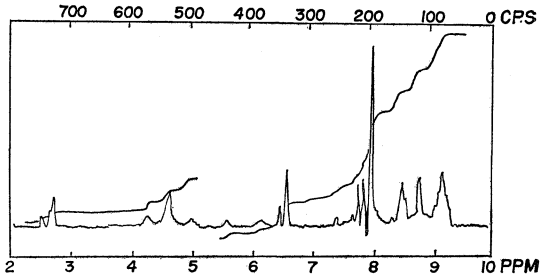
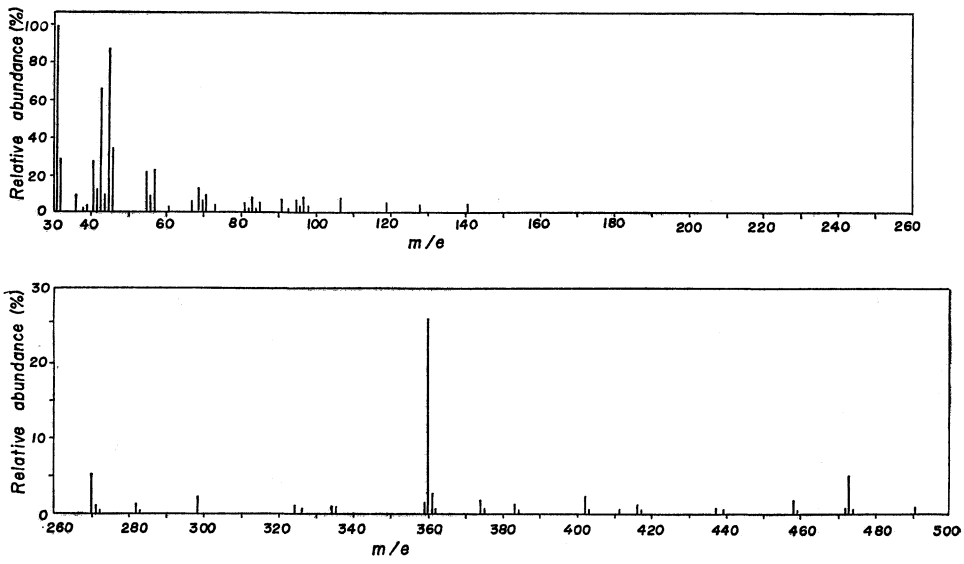


Fig. 5. Mass spectrum of funiculosin



The presence of amide grouping was suggested by bands at 3600~3000, 1649 and 1560 cm^{-1} in the infrared absorption spectrum (IR, Fig. 3) a signal of one proton attached to amide nitrogen at τ 2.53 in $CDCl_3$ (Fig. 4), where the acetyl funiculosin was used instead of funiculosin because the derivative is more soluble in chloroform. The presence of alcoholic hydroxyl groupings was evident by bands at 3600~3000, 1148, 1128, 1103 and 1039 cm^{-1} in IR. Thus, we concluded that funiculosin is a new antibiotic.

Experimental

1. Fermentation: The spores of *P. funiculosum* were inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a medium composed of (w/v, %); glucose 2, lactose 2, corn steep liquor 3, $NaNO_3$ 0.1, KH_2PO_4 0.06, KCl 0.05, $MgSO_4 \cdot 7H_2O$ 0.02 and $CaCO_3$ 1 in tap water. The pH of the medium was adjusted to 6.0~6.2 before addition of $CaCO_3$. The flasks were incubated at 27°C for 6 days on rotary shaker. Three hundred ml of the fermented broth thus prepared was transferred to a jar fermentor of 100-liter in volume contaning 60 liters of the medium with the same composition as that of the inoculation medium. The fungus was grown at 27°C for 4 days under an aeration rate of 30 liters per minute and an agitation speed of 300 rpm. The mycelium obtained by centrifugation of the fermented broth was extracted with acetone (20 liters) overnight at room temperature. After filtering the mycelium, the acetone extract was concentrated *in vacuo* to remove acetone. The residual suspension (5 liters) was extracted twice with ethylacetate (3 liters × 2) and the combined extracts were dried over anhydrous sodium sulfate. After removing the sodium sulfate, the filtrate was concentrated *in vacuo* to a small volume. Residual oil contained 1.2 g of funiculosin on the basis of antifungal activity against *C. albicans*.

2. Isolation of funiculosin: The oil was fractionated through a silica gel column of 36 mm in diameter and 1,000 mm in length packed with a mixture of silica gel (Mallinkrodt, 100 mesh, 80 g) and celite 545 (20 g) suspended in benzene. The column was washed with benzene (500 ml) and then eluted with benzene-methanol (95 : 5, 1.5 liter). The antifungal fractions inhibiting *C. albicans* were combined and concentrated *in vacuo* to thick syrup. Funiculosin (620 mg) crystallized as needles when the syrup was kept at 5°C overnight. The antibiotic was recrystallized from ethylacetate, mp 154~155°C (decomp.), molecular weight 491 determined by mass spectroscopy.

Anal. Calcd. for $C_{27}H_{41}NO_7$: C 65.99, H 8.35, N 2.83.
Found: C 65.49, H 8.66, N 2.57.

3. Antiviral activity: The method previously reported was used[8]. The virus-cell systems used were CEF-NDV, herpes simplex virus and vaccinia virus in the agar-diffusion method and HeLa cells-herpes simplex virus and vaccinia virus in the tube culture method.

4. Antitumor activity: The mouse strain *ddY*-EHRLICH ascites tumor system was used. Funiculosin is insoluble in water so that 0.1 % of Tween 80 was included in the phosphate buffer saline (pH 7.0) in suspending the antibiotic.

5. Measurement of spectra: Mass spectrum was taken by Hitachi RMU-6E mass spectrometer. Japan Electron Optics JNM-3H-100 nuclear magnetic resonance spectrometer was used for measurements of NMR spectra in $CDCl_3$ using TMS as an internal standard. IR spectra were recorded by Japan Spectroscopic Co. Model IR-S. Ultraviolet absorption spectra were obtained by Cary Model 11-M.

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