VIRANTMYCIN, A NEW ANTIVIRAL ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES*

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Virantmycin, a novel chlorine-containing antiviral antibiotic, has been isolated from *Streptomyces nitrosporeus* No. AM-2722. The active substance in culture broth is isolated as colorless needles by solvent extraction followed by high performance liquid chromatography on silicic acid. The molecular formula is $C_{19}H_{28}NO_3Cl$ (molecular weight 351) from the elemental analysis and mass spectrum. The antibiotic possesses antifungal activity and potent inhibitory activity against various RNA and DNA viruses.

In the course of our screening program for IFN (interferon) inducers and antiviral substances from *Streptomyces*, we found that *Streptomyces* sp. No. AM-2722, isolated from the putrefied tissue portion of a pumpkin, produces a novel antibiotic possessing a potent inhibitory activity against both RNA and DNA viruses.[1] The antibiotic also shows weak antifungal activity. The antibiotic, named virantmycin, is a novel antiviral antibiotic containing chlorine. The structure of virantmycin has been reported elsewhere.[2] In this paper, we describe the taxonomy of the producing strain, and the production, isolation and physicochemical and biological properties of virantmycin.

Taxonomic Studies

Strain AM-2722 was isolated from a pumpkin spoiled by microorganisms. The strain was cultivated at 27°C for 14 days on various media such as yeast extract-malt extract agar, inorganic salts - starch agar, glucose - asparagine agar and tyrosine agar. Its morphological characteristics were examined with both optical and electron microscopes. The sporophores are *rectus-flexibilis* type and have more than ten spores per chain. Conidia with smooth surface are oval ($0.4 \sim 0.5 \times 1.0 \sim 1.1\ \mu$) as shown in Fig. 1. No sclerotic granules, sporangia or zoospores were observed. The characteristics of cultures incubated at 27°C for 14 days are shown in Table 1. The physiological properties and utilization of carbon sources are summarized in Tables 2 and 3, respectively. The color terms recorded for each culture are those of the Color Harmony Manual.[3] The cell wall of strain AM-2722 contains LL-diaminopimelic acid (DAP). This and the morphological characteristics described above indicate that the strain belongs to genus *Streptomyces*.

Its properties are summarized as follows: Sporophore morphology, *rectus-flexibilis*; mature spore chains, more than 10 spores per chain; spores, oval form with smooth surface; aerial mass color, white and/or gray; vegetative mass color, yellow and/or gray; melanoid and soluble pigment, none; utilizable carbon, D-glucose, L-arabinose, D-xylose, and rhamnose; DAP in cell wall, LL-type; whole cell sugar pattern, no characteristic pattern.

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Table 1. Cultural characteristics of strain AM-2722.

Glucose-nitrate agar	G: thin, pearl (3ba) R: pearl (3ba) AM: poor, velvety, natural (3dc) SP: none
Sucrose-nitrate agar	G: moderate, colorless R: colorless AM: moderate, velvety, ivory tint (2cb) SP: none
Glycerol-calcium-malate agar	G: moderate, flat and penetrate, colorless R: colorless AM: poor, velvety, white~gray (a~g) SP: none
Glucose-asparagine agar	G: good, slightly raised, bamboo (2gc) R: bamboo (2gc) AM: abundant, velvety, ivory tint (2cb) SP: none
Glycerol-asparagine agar (ISP)*	G: good, slightly raised, yellow tint (1ba) R: covert brown (2nl) AM: abundant, powdery, gray (d) SP: none
Inorganic salts-starch agar (ISP)*	G: good, slightly raised, colorless R: colorless AM: abundant, velvety, gray (d) SP: none
Tyrosine agar (ISP)*	G: good, penetrate, pearl pink (3ca) R: gray (f) AM: abundant, velvety, gray (d) SP: light brownish gray (5cb)
Yeast extract-malt extract agar (ISP)*	G: good, wrinkled, raised, light ivory (2ca) R: light ivory~covert brown (2ca~2li) AM: abundant, water drop, velvety, gray (b~d) SP: none
Oatmeal agar (ISP)*	G: moderate, flat, colorless R: colorless AM: moderate, velvety, gray (h) SP: none
Peptone-yeast extract iron agar (ISP)*	G: thin, bamboo (2gc) R: bamboo (2gc) AM: none SP: none
Glucose-peptone agar	G: moderate, raised, cream (1½ca) R: cream (1½ca) AM: moderate, velvety, white (a) SP: none
Nutrient agar	G: good, penetrate, light wheat (2ea) R: light wheat (2ea) AM: moderate, velvety, white (a) SP: none

Abbreviations used in Table 1: G, growth; R, reverse; AM, aerial myceliun; SP, soluble pigment.
* Medium employed by the International Streptomyces Project.

Among the known species of *Streptomyces* described in "BERGEY's Manual of Determinative Bacteriology" 8th ed.[4] and WAKSMAN's "The Actinomycetes" Vol. II,[5] it was concluded that strain AM-2722 was a strain of *Streptomyces nitrosporeus*.[6] The strain has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as *Streptomyces* sp. AM-2722

Fig. 1. Electronmicrograph of the conidia of strain AM-2722.

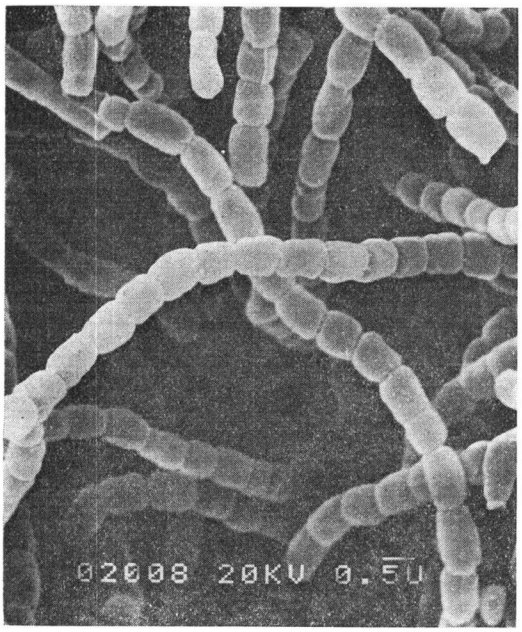


Table 2. Physiological properties of strain AM-2722.

Melanin formation	−
Tyrosinase reaction	−
H_2S Production	−
Nitrate reduction	+
Liquefaction of gelatin	−
Hydrolysis of starch	+
Coagulation of milk	−
Peptonization of milk	+
Cellulolytic activity	−
Temperature range of growth	22°C~34°C

Table 3. Utilization of carbon sources by strain AM-2722.

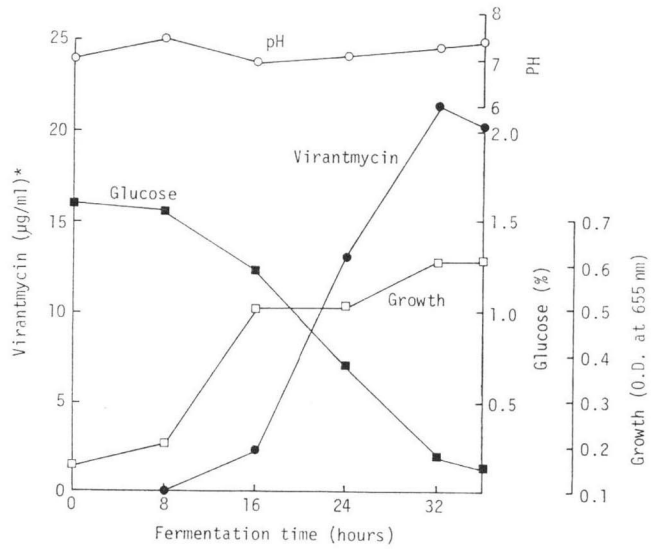
Positive	D-glucose, L-arabinose, D-xylose, rhamnose
Doubtful	sucrose, D-fructose, D-mannitol
Negative	*i*-inositol, raffinose

with the accession number FERM-P 5419.

Antibiotic Production

A well sporulated agar slant of *Streptomyces nitrosporeus* No. AM-2722 was used as a pre-seed culture. The cultivation medium containing 2% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dry yeast, 0.5% NaCl, 0.3% $CaCO_3$ in shaking flasks was incubated for the seed culture at 27°C on a rotary shaker (180 r.p.m.) for 2 days. Fermentation was carried out in a 100-liter fermentor containing 70 liters of the above medium at 27°C with aeration at 30~35 liters per minute and agitation at 200 r.p.m.

Fig. 2. Virantmycin production by *Streptomyces nitrosporeus* AM-2722.



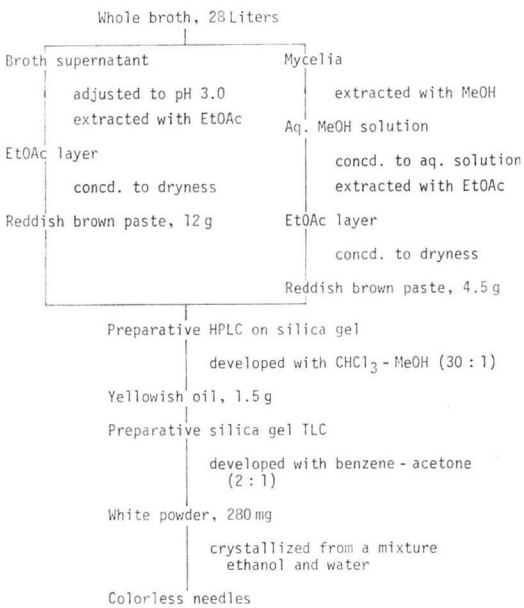
* The antibiotic was assayed by analytical HPLC.

The antibiotic was assayed by the plaque reduction method with vesicular stomatitis virus (VSV) as an indicator and detected chemically on silica gel thin-layer chromatograms (Kiesel gel 60 F_{254}) of the solvent extract from the whole broth. The virantmycin content in the whole broth was determined by using analytical liquid chromatography with a μBondapak C_{18} column (solvent: MeOH - H_2O - HOAc, 80: 20: 5 (v/v), flow rate: 1 ml/minute, detector: UV at 300 nm) for the ethyl acetate extract from the whole broth at pH 3. The time course of virantmycin production detected by the application of analytical liquid chromatography is shown in Fig. 2. The production of virantmycin in the fermentation broth reached a maximum at about 40 hours and decreased moderately beyond 40 hours.

Isolation and Purification

The isolation procedure for virantmycin is summarized in Scheme 1. The broth was acidified with conc. hydrochloric acid to pH 3.0 and centrifuged with the aid of a Sharpless centrifuge. The wet mycelial cake was suspended in methanol with stirring. The methanol extract was concentrated *in vacuo* to a brownish syrup. On the other hand, the broth supernatant was extracted twice with ethyl acetate. The extract was concentrated *in vacuo* to dryness to obtain an oily substance. The crude materials obtained from the broth supernatant and the mycelia were combined. A solution of the crude material in the solvent mentioned below was subjected to preparative liquid chromatography (Prep LC/System 500) on silica gel (2 packed silica gel columns, 300 g×2) using a mixture of chloroform and methanol (30: 1, v/v) with flow rate at 250 ml/minute under 30 atmospheres. Elution of virantmycin was followed by examining the anti-VSV activity and by detection on a silica gel thin-layer plate (Kiesel gel 60 F_{254}, developer; benzene - acetone, 2: 1, v/v, Rf 0.05) with ferric chloride or by UV absorbance. The active eluates were combined and concentrated *in vacuo* to give a dark brownish oil. Further purification of the antibiotic was carried out by a preparative silica gel thin-layer chromatography (Kiesel gel 60 F_{254}, developer; benzene - acetone, 2: 1, v/v). The pure material of antibiotic was crystallized from a mixture of ethanol and water to obtain colorless needles.

Scheme 1. Isolation procedure of virantmycin.



Physicochemical Properties

Virantmycin is soluble in benzene, acetone, chloroform, ethyl acetate, ethyl ether, methanol, ethanol, slightly soluble in *n*-hexane, and practi-

Table 4. Physicochemical properties of virantmycin.

Appearance	colorless needles
mp.	59°C
$[\alpha]_D^{18}$	$-0.05°$ (*c* 1, $CHCl_3$)
Mol. formula	$C_{19}H_{26}NO_3Cl$
Elemental analysis (%)	C, 65.1; H, 7.1; N, 3.7; Cl, 10.3
Mass	M^+ *m/z* 351, 351.161 calcd. for $C_{19}H_{26}NO_3Cl$ 351.160 *m/z* 316 (M^+-Cl), *m/z* 306 ($M^+-CH_2OCH_3$), *m/z* 270 ($M^+-C_2H_6OCl$), *m/z* 254 ($M^+-C_7H_{13}$), *m/z* 83 (C_6H_{11})
UV λ_{max}^{EtOH} nm (ε)	226 (3500), 306 (8100)
IR (CCl_4) cm^{-1}	3440 (NH), 3400~2400 (OH), 1690 (CO), 1603 (C=C)

cally insoluble in water. The antibiotic gives positive reaction to DRAGENDORFF, ferric chloride and bromocresol green reagents, but negative to MOLISH and LEGAL. The BEILSTEIN reaction is positive suggesting the presence of a chlorine atom in the molecule.

Some physicochemical properties of virantmycin are listed in Table 4. Virantmycin forms colorless needles, mp. 59°C, $[\alpha]_D^{18}$ −0.05° (*c* 1, $CHCl_3$). The molecular formula, $C_{19}H_{26}NO_3Cl$ was established by an accurate mass measurement of the fragment ion in the high resolution mass spectrum [M^+ *m/z* 351, fragment ion *m/z* 316 (M^+-Cl), Found 316.1912; Calcd. for $C_{19}H_{26}NO_3$, 316.1925] and its elemental analysis. The mass spectrum clearly indicated the presence of a chlorine atom in the molecule. The UV spectrum (Fig. 3) in ethanol exhibited an absorption maxima at 226 nm (ε, 3500) an 306 nm (ε, 8100).

Fig. 3. UV spectrum of virantmycin (in EtOH).

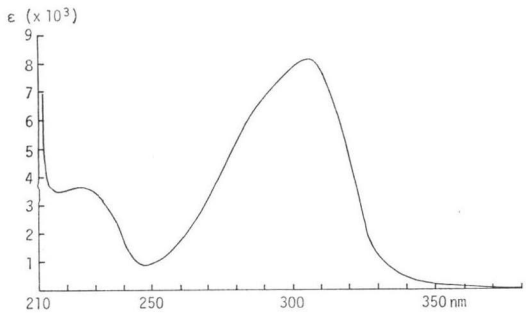


Table 5. Antiviral activity of virantmycin against several RNA and DNA viruses.

Virantmycin concentration (μg/ml)	% of plaque reduction			
	VSV	NDV	WEE	SbV
10	100	100	100	100
1	98	100	100	100
0.1	97	85	100	100
0.01	68	25	92	85
0.001	1	0	27	0
	Vac.-DIE	Vac.-IHD	HSV-1	HSV-2
10	100	100	100	100
1	99	100	100	100
0.1	99	100	99	99
0.01	94	86	28	37
0.001	0	23	0	0

Plaque reduction test of virantmycin on several RNA and DNA viruses was carried out as follows. Primary CE cells were prepared as described in Materials and Methods. Cells were treated with 0.001 ~ 10 μg/ml of virantmycin at 37°C for 20 hours. Virantmycin was removed and cells were infected with 100 PFU/dish of each virus. Afterthere, cell cultures were subjected to the method of *plaque formation*[7]. Percentage of plaque reduction was calculated by following formula:

$$\frac{\text{Plaque count (control)} - \text{Plaque count (virantmycin)}}{\text{Plaque count (control)}} \times 100$$

The IR spectrum in carbon tetrachloride showed absorption bands characteristic of a carboxylic acid (ν_{NH} 3440 cm^{-1} and ν_{CO} 1690 cm^{-1}) and chlorine (627 cm^{-1}). The ^1H NMR spectrum in $CDCl_3$, as shown in Fig. 4 showed the presence of three *tert*-methyl groups (δ 1.62), a methoxy group (δ 3.4), three aromatic protons (δ 6.56, 7.78 and 7.82) and a carboxyl proton (δ 8.0). The ^{13}C NMR spectral analysis of virantmycin indicated the existence of a benzoic acid skeleton, a double bond, a methoxymethylene, three methylenes, three methyls, a methine and a quarternary carbon. The structure of the antibiotic has been determined by chemical degradation and spectrometric means, as shown in Fig. 5.

Biological Properties

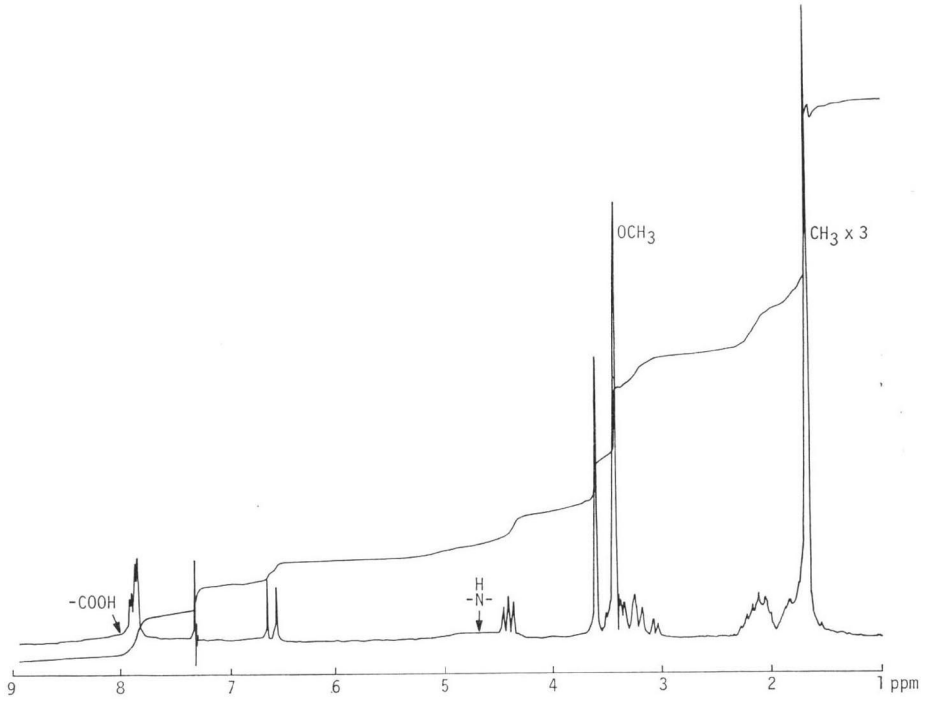
The antiviral activity of the antibiotic was assayed by a plaque reduction test.

Materials and Methods

Primary chick embryonic (CE) cells were prepared by trypsinizing 9 days old chick embryos and cultivated in 3 ml of minimum essential medium (MEM) supplemented with 10% calf serum (CS) in 40 mm glass dishes. Rabbit continuous cell line RK-13 cells were also cultivated with MEM-CS 10%.

Egypt Ar 339 strain of Shindbis virus (SbV), MCMILLAN strain of Western equine encephalitis virus (WEE), DIE strain of vaccinia virus (Vac-DIE), IHD strain of vaccinia virus (Vac-IHD), HF strain of

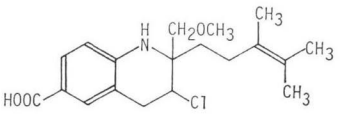
Fig. 4. ^1H NMR spectrum of virantmycin (100 MHz, in $CDCl_3$).



herpes simplex virus type 1 (HSV-1), and UW strain of herpes simplex virus type 2 (HSV-2) were given by Dr. M. TOBA (Institute of Medical Sciences, University of Tokyo). Indiana strain of vesicular stomatitis virus (VSV) and MIYADERA strain of Newcastle disease virus (NDV) were propagated as described previously.[7]

Plaque formation of viruses and plaque reduction test of virantmycin were carried out as described previously.[1]

Fig. 5. Structure of virantmycin.



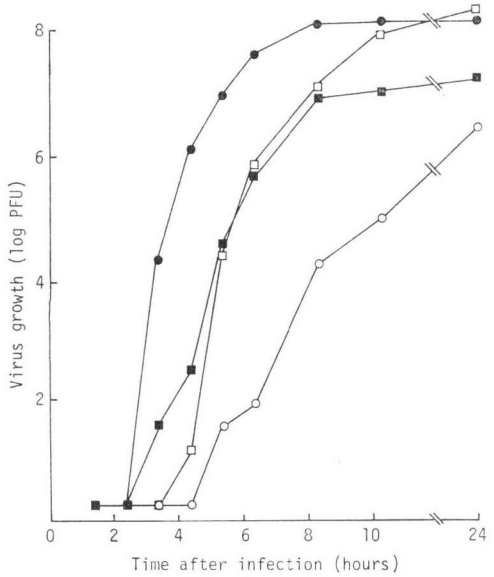
Results and Discussions

As seen in Table 5, virantmycin inhibited plaque formation by several RNA and DNA viruses at low concentrations. It should be noted that virantmycin inhibits the growth of both RNA and DNA viruses which differ from each other in their growth mechanism. Furthermore, it is a question of interest as to whether the mechanism of action as the same for both types of virus.

The influence on the one step growth of VSV in RK-13 cells treated with virantmycin was investigated as follows.[8] Monolayers of RK-13 cells were incubated in the presence or absence of 10 μg/ml of virantmycin in MEM CS 2% at 37°C for 20 hours (pre-treatment). Medium was removed and the cells were infected with VSV at an input of multiplicity of infection (m.o.i.), 10 PFU (plaque forming unit) per cell. Ten μg/ml of virantmycin was added to some cultures at the same time and cells were incubated for 1 hour. The inocula were removed and the infected cells were washed three times with phosphate-buffered saline and fed with fresh MEM CS 2% with or without 10 μg/ml of virantmycin (post-treatment). Cells were further incubated and the cultured fluids were aliquoted at indicated times to assay the growth of virus in each culture.

Representative results are shown in Fig. 6. In the control culture to which no virantmycin was

Fig. 6. Influence of virantmycin on one step growth of VSV in RK-13 cells.



Virus infectivities from virantmycin-treated or untreated control cultures at times indicated are plotted. Cells were infected with VSV at m.o.i. 10 at 0 time. Virantmycin (10 μg/ml) was added to each culture medium from −20 hours to 0 hour (□—□, pre-treatment), from 0 hour to 24 hours (■—■, post-treatment), and from −20 hours to 24 hours (○—○, pre- plus post-treatment). Control culture is shown as (●—●).

Table 6. Antifungal activity of virantmycin.

Test organism	MIC (μg/ml)
Candida albicans	50
Saccharomyces sake	25
Piricularia oryzae	25
Trichophyton interdigitale	25
Aspergillus niger	12.5
Alternaria kikuchiana	25
Mucor racemosus	25

Potato glucose agar, 27°C, 2~4 days.

added, a typical profile of one step growth of VSV was seen; no virus was released into cultured fluid till 3 hours after virus infection, and thereafter, virus release was first detected at 3 hours, increased up to the 8th hour and reached a maximum level of cumulative titers of virus infectivities. In the virantmycin-pre-treated culture, on the other hand, delay and reduction of virus release were observed upto the 8th hour as compared to the control and virus release came up the level with the control after further incubation. In the virantmycin-post-treated culture, virus release profile was similar to the pre-treated culture up to the 8th hour but significant reduction removed throughout further incubation. In the virantmycin pre-plus post-treated culture, much more reduction in virus release was observed. From these results, virantmycin apparently inhibits the one step growth of VSV. Both pre- and post-treatment of the antibiotic showed suppression of the growth of the virus, however, the persistency of the antibiotic action seemed to be no longer than about 8 hours after the antibiotic was removed.

Virantmycin also posseses weak antifungal activity. The minimum inhibitory concentration (μg/ml) of the antibiotic is shown in Table 6.

Acknowledgement

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