TUNICAMYCIN, A NEW ANTIBIOTIC. I

ISOLATION AND CHARACTERIZATION OF TUNICAMYCIN

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(Received for publication December 3, 1970)

Tunicamycin is a new antiviral antibiotic produced by *Streptomyces lysosuperificus*, nov. sp. The active substance in culture broth is purified by solvent extraction followed by chromatography on silicic acid. It has a molecular weight of about 870. The antibiotic is active against animal and plant viruses, Gram-positive bacteria, yeast and fungi. Tunicamycin is similar in some properties to a few known antibiotics but differs from them in certain special characteristics.

Employing the agar diffusion method of HERRMANN *et al.*[1], antiviral substances have been searched for among microbial products. Among about 4,000 Actinomycetes isolated from soil samples, one strain was found to produce a new antibiotic active against both RNA and DNA viruses. The active principle was isolated and characterized. Effects of the antibiotic on incorporation of precursors of cell constituents suggest that it acts on membrane synthesis in animal cells and alters some other synthetic processes including nucleic acids and protein (manuscript in preparation). Morphological changes were induced by the antibiotic in sensitive microorganisms including bacteria and yeasts without inhibition of protein and nucleic acid syntheses (unpublished observations). The antibiotic was named tunicamycin (*tunica* means coat) after its mode of action. The producing organism was also found to be a new species of the genus *Streptomyces* and named *Streptomyces lysosuperificus* nov. sp. after its characteristics of growth and the action mechanism of the antibiotic it produces. The taxonomic studies of the producing organism will appear in a subsequent paper of this series. Production, isolation, and chemical, physical and biological properties of tunicamycin will be presented in this paper.

I. Production and Isolation of Tunicamycin

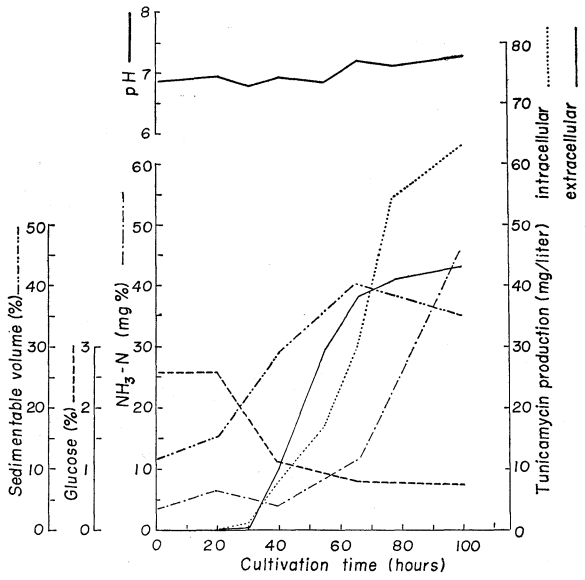
(1) Production by fermentation

Streptomyces lysosuperificus was cultivated in a medium containing 2% glucose, 2% gluten meal, 2% Pharmamedia, 0.5% Ebios, 1.5% KH_2PO_4, 3% K_2HPO_4 and 0.3% $CaCO_3$. Culture was carried out at 27°C on a shaking machine, in a jar fermentor or in a tank fermentor with adequate aeration and agitation. Growth of the organism was very good and culture broth was very foamy because of lysis of the organism. The lysis could not be prevented in any producing medium tested.

An example of the fermentation process is shwon in Fig. 1. Tunicamycin was detected after 30 hours and increased throughout the cultivation for 100 hours.

Tunicamycin was found to be stable even when lysis of the producing organism had taken place. Antibiotic activity was checked by paper-disc bioassay with Newcastle disease virus (NDV) as an indicator organism.

Fig. 1. A time course of fermentation of tunicamycin.



(2) Isolation

Tunicamycin could be isolated by various combinations of well known methods. A representative flow sheet of the isolation is shown in Fig. 2.

The culture broth of *Streptomyces lysosuperificus* was filtered with the aid of Celite 545 (Johns-Manville). The mycelial cake was extracted with methanol two times. The combined methanol extract was concentrated to remove the solvent at below 45°C *in vacuo*. The concentrate was adjusted to pH 3 with HCl and stirred with *n*-butanol. The *n*-butanol extract was concentrated to a syrup. Three to five volumes of acetone was added to the syrup and the mixture was held at 4°C to precipitate tunicamycin. Precipitates were collected by filtration or centrifugation and dissolved in alkaline water. The water solution was washed once with ethylacetate after adjusting the pH to 3 followed by extraction with *n*-butanol two to three times. Washing with *n*-butanol at alkaline pH removed gummy materials to a great extent but much of the antibiotic was lost in the discarded butanol. The *n*-butanol extract was concentrated *in vacuo*. The crude extract was fractionated by passing through a silicic acid (Mallinckrodt) column eluted with a chloroform-methanol mixture (1 : 1~1 : 2). Elution of tunicamycin was followed by examining the anti-NDV activity of each fraction. Active fractions were collected and concentrated to a small volume. The concentrate was held at 4°C overnight to obtain tunicamycin as a crystalline powder. A small amount of acetone was added in some cases to promote the precipitation. The recovered precipitate was further purified by recrystallization from methanol, methanol - acetone or methanol - ethanol mixture.

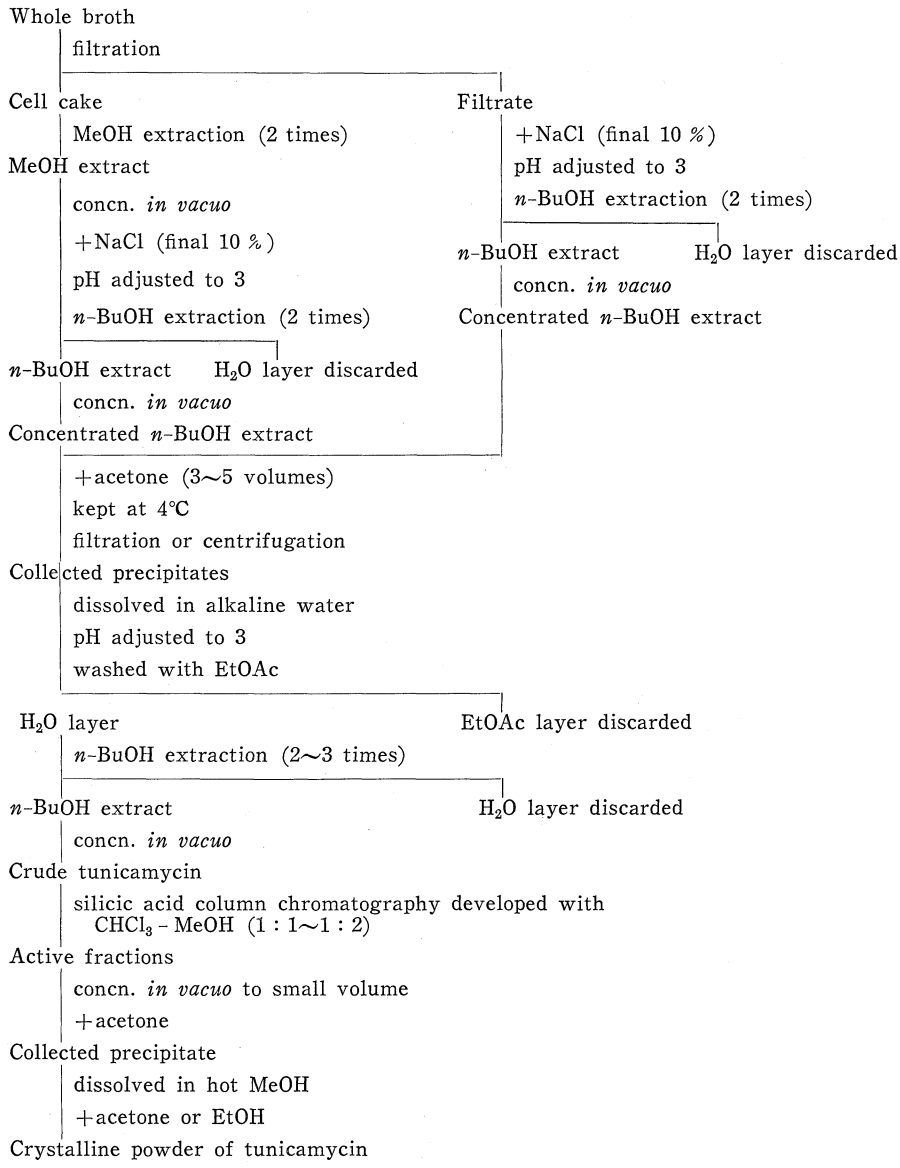
Tunicamycin was also obtained from the culture filtrate by a similar procedure.

II. Physical and Chemical Properties

Pure tunicamycin was obtained as a white crystalline powder with physical and chemical properties as follows:

(1) Solubility: Soluble in alkaline water, pyridine and hot methanol, slightly soluble in ethanol and butanol, insoluble in acetone, ethylacetate, chloroform, benzene and acid water.

Fig. 2. Extraction and isolation procedures of tunicamycin.



(2) Melting point: 234~235°C (decomp.)

(3) Optical rotation: $[\alpha]_D^{20}$ +52° (*c* 0.5, pyridine)

(4) Color reaction: Positive I_2, $AgNO_3$, $KMnO_4$ and RYDON-SMITH reactions. Negative ninhydrin, SAKAGUCHI, ELSON-MORGAN, diazo, $FeCl_3$, FEHLING, aniline phthalate and hydroxylamine-Fe reactions.

(5) Thermostability: When tunicamycin was dissolved in water and held at 100°C for 30 minutes, it was stable at neutral and alkaline pH, but unstable at acidic pH.

(6) Molecular weight: Tunicamycin did not give a satisfactory mass spectrum. Molecular weight determinations by the vapor pressure equilibrium method (Model

115 Hitachi) gave a value of 870.

(7) Elementary analysis: Found: C 52.59, H 7.67, N 6.25. Calculated for $C_{38}H_{66}N_4O_{18}$ (M. W. 866.94): C 52.53, H 7.67, N 6.46. The above molecular formula of tunicamycin is a tentative one that requires further studies.

(8) Ultraviolet absorption spectrum (Fig. 3): Tunicamycin showed two peaks of absorption at 205 mμ ($E^{1\%}_{1cm}$ 230) and 260 mμ ($E^{1\%}_{1cm}$ 110) in methanol.

(9) Infrared absorption spectrum (Fig. 4): The presence of -OH and/or -NH (3400 and 3300

Fig. 3. UV spectrum of tunicamycin in methanol.

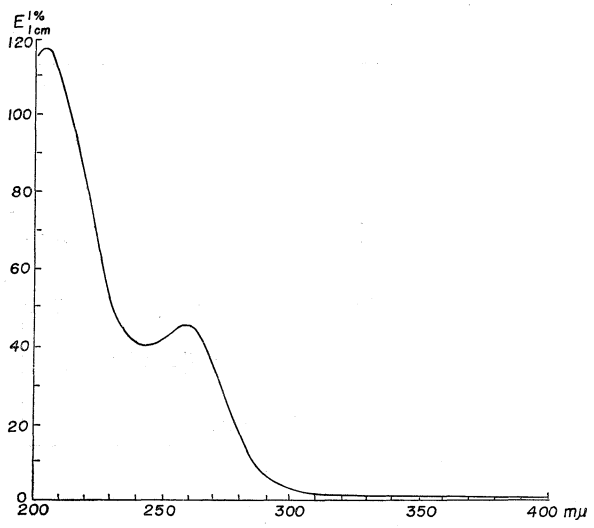


Fig. 4. IR spectrum of tunicamycin (Nujol mull).

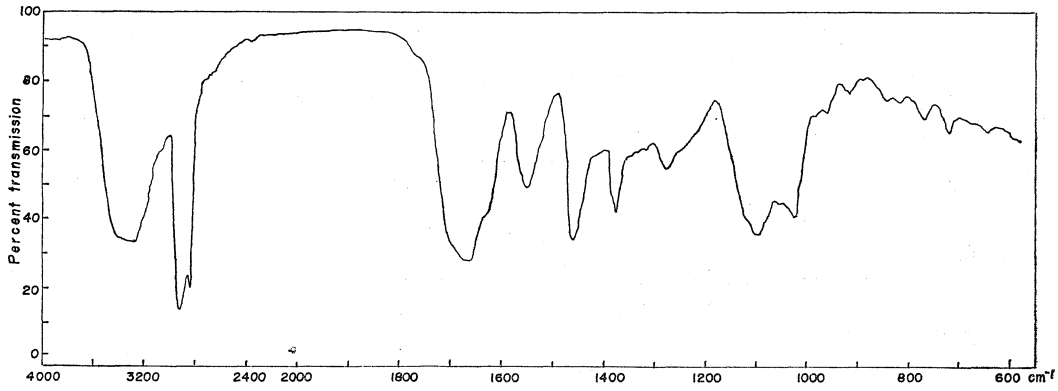


Fig. 5. NMR spectrum of tunicamycin in $C_5D_5N_1$.

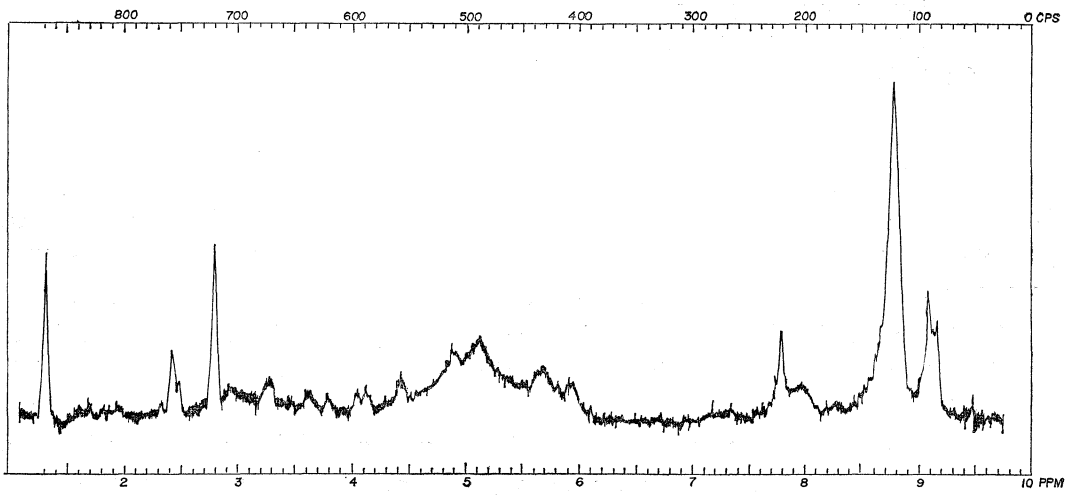


Fig. 6. Thin-layer chromatograms of tunicamycin (T) and mycospocidin (M).

Spot films of silica gel and alumina were obtained from Tokyo Kasei Co., Tokyo. Cellulose is a product of Funakoshi Yakuhin Co., Tokyo.

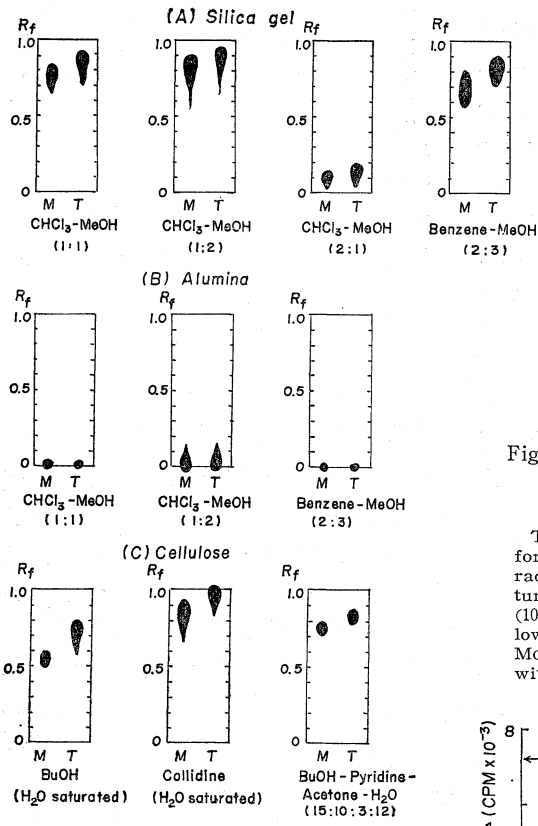


Fig. 7. Time course of hydrolysis of tunicamycin with 2 N HCl.

Tunicamycin (2 mg/ml) was hydrolyzed with 2 N HCl at 105℃. At designated time intervals, ELSON-MORGAN reaction of the hydrolysates was carried out. Measurements of O.D.$_{535\,m\mu}$ were expressed as molar glucosamine equivalents from one mole tunicamycin (M.W. 870).

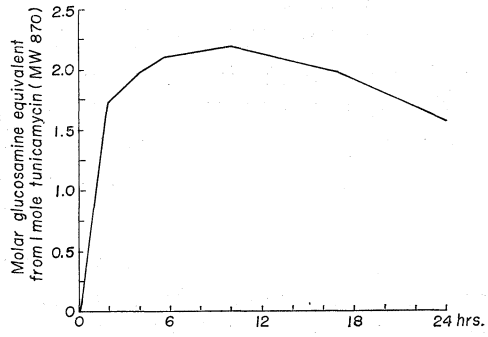
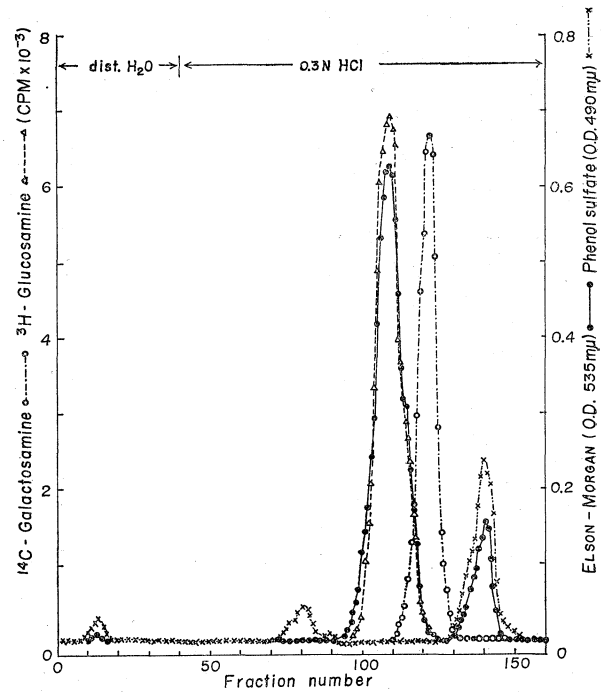


Fig. 8. Co-column chromatography on Dowex 50 (H^+ type) of tunicamycin hydrolysate, ^{14}C-galactosamine and ^{3}H-glucosamine.

Tunicamycin was hydrolyzed with 2 N HCl at 105℃ for 16 hours. After neutralization, it was mixed with radioactive glucosamine and galactosamine. The mixture was passed through Dowex 50 (H^+ type) column (10×500 mm) developed with distilled water, and followed with 0.3 N HCl. Radioisotope counting, ELSON-MORGAN and phenol-sulfate reactions were carried out with each fraction (3 ml).



cm^{-1}) and secondary amide (1690, 1550 and 1280 cm^{-1}) groups was indicated.

(**10**) Nuclear magnetic resonance spectrum (Fig. 5): NMR spectrum of tunicamycin dissolved in $C_5D_5N_1$ is presented. When NMR was measured with tunicamycin dissolved in methanol, no aromatic hydrogen absorption was observed.

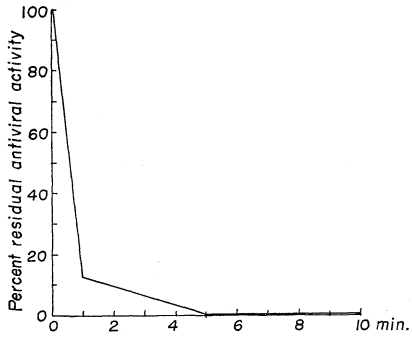
(**11**) Chromatography (Fig. 6): The chromatograms of tunicamycin and mycospocidin, an antibiotic with characteristics similar to tunicamycin as will be discussed below, are presented. The antibiotics were visualized by bioautography. Tunicamycin is differentiated from mycospocidin in

water-saturated *n*-butanol on cellulose.

(12) Hydrolysis of tunicamycin: Hydrolysis of tunicamycin was carried out with 2 N HCl, 2 N H_2SO_4 or 2 N KOH. Tunicamycin is resistant to alkaline hydrolysis and antiviral activity is detected even after a 24-hour treatment with 2 N KOH at 105°C. On the other hand, tunicamycin is easily hydrolyzed with acids, especially with HCl, and hydrolysis with 2 N HCl was nearly complete in 2 hours as revealed by ELSON-MORGAN reaction (Fig. 7). Analysis of an acid hydrolysate of tunicamycin (6 N HCl, 16 hours at 105°C) with an amino acid analyzer showed the presence of glucosamine but not of any amino acid. The presence of glucosamine in the hydrolysate of tunicamycin was confirmed by co-column chromatography on Dowex 50 (H^+ type) with radioactive glucosamine and galactosamine (Fig. 8). The major peak with positive ELSON-MORGAN reaction was eluted with the radioactive glucosamine. A second peak was detected after the radioactive galactosamine. The second peak gave positive ELSON-MORGAN reaction, but it showed stronger phenol-sulfate reaction than glucosamine. Treatment of glucosamine with 2 N HCl at 105°C for more than 24 hours and column chromatography on Dowex 50 did not exhibit such a peak. Therefore, the second peak can not be attributable to some degradation product of glucosamine itself. Identification of the second peak is now in progress.

Fig. 9. Treatment of tunicamycin with periodate.

Tunicamycin (2 mg/ml) was treated with 0.01 M sodium periodate at room temperature in the dark. The reaction was stopped by adding an aliquot of the reaction mixture into ethyleneglycol solution at designated time intervals. Residual antiviral activity was determined by the agar diffusion method employing NDV and chick embryo cells.



No neutral sugar was detected by phenol-sulfate reaction on column chromatography on Dowex 50 (H^+ type) eluted with distilled water as shown in Fig. 8. The ELSON-MORGAN reaction of the hydrolysate of tunicamycin mostly depends upon glucosamine (Fig. 8). It is concluded from the result shown in Fig. 7 that tunicamycin contains two moles glucosamine in its molecule.

(13) Treatment of tunicamycin with periodate: Treatment with periodate promptly and completely destroyed the antiviral activity of tunicamycin (Fig. 9). This result suggests that sugar moiety of tunicamycin is essential for the antivial activity of the antibiotic. Reversal of the antiviral activity of tunicamycin with some amino sugars[2] also indicates the important role of the sugar moiety of tunicamycin in its antivral activity.

III. Biological Properties

(1) Antiviral activity: Tunicamycin inhibited multiplication of NDV and herpes simplex virus in cultured cells to nearly the same extent (Table 1), and thus both RNA and DNA viruses are included in its antiviral spectrum. Some biological aspects of the antiviral activity of tunicamycin will be presented in a subsequent paper of this series[3].

Table 1. Cytotoxicity and antiviral activity of tunicamycin as determined by the agar-diffusion plaque-inhibition method

	mcg/ml
Maximum concentration tolerated by cultured chick embryo fibroblasts	>12,000
Minimum inhibitory concentration against NDV	0.5
HSV	0.5

The Miyadera strain of NDV and the HF strain of herpes simplex virus were used. Two-day cultures of chick embryo fibroblasts in Petri dishes were employed as host cells.

Table 2. Anti-TMV activity of tunicamycin as determined by the local lesion method.

Concentration (mcg/ml)	Inhibition ratio (%)	Phytotoxicity
100	100	+
20	100	−
4	100	−
0.8	100	−
0.16	100	−
0.032	52	−
0.0064	0	−

Coleoptiles of 2-week-old *Phaseolus vulgaris* L., Pinto were infected with TMV with an aide of carborundum. Leaf discs (11 mm in diameter) were prepared from the infected coleoptiles and were floated on antibiotic solutions in Petri dishes. After an incubation at 25℃ under continuous 3,000 lux fluorescent illumination, local lesions formed on the leaf discs following TMV multiplication were counted. Effect of tunicamycin on TMV multiplication was expressed as % inhibition of the occurrence of local lesions. Phytotoxicity of the antibiotic on the leaf discs was observed at the same time.

Tunicamycin suppressed occurrence of local lesions on tobacco mosaic virus (TMV)-infected leaf discs (Table 2). It also inhibited multiplication of TMV *in vivo* in leaves of *Nicotiana tobacum* (unpublished observation). Slight toxicity was observed at higher concentrations than that required for inhibition.

(2) Antimicrobial activity: The minimum inhibitory concentration of tunicamycin against a variety of microorganisms was examined by tube or paper disc methods. The results are given in Table 3. Tunicamycin was active against Gram-positive bacteria, yeasts and fungi. *Bacillus* species were the most sensitive to the antibiotic among all examined. Activity against *Piricularia oryzae* was nearly the

Table 3. Antimicrobial spectrum of tunicamycin as determined by tube dilution method

The organisms		Minimum inhibitory concentrations (mcg/ml)	The organisms		Minimum inhibitory concentrations (mcg/ml)
Pseudomonas ovalis	IAM 1091	>100	*Bacillus subtilis*	IAM 1028	2
Pseudomonas aeruginosa	IAM 1095	>100	*Bacillus cereus*	IFO 1110	2
Aerobacter aerogenes	IAM 1019	>100	*Bacillus alvei*	IAM 1258	10
Proteus vulgaris	IAM 1025	>100	*Bacillus firumus*	IAM 1188	5
Escherichia coli K-12		>100	*Bacillus roseus*	IAM 1257	20
Escherichia coli B		>100	*Bacillus thiaminolyticus*	IAM 1034	5
Azotobacter agile	IAM 1078	25	*Bacillus aneurinolyticus*	IAM 1077	5
Achromobacter cycloclastes	IAM 1013	>100	*Bacillus stearothermophilus*	IAM 1035	5
Flavobacterium aquatil	IFO 3772	100	*Mycobacterium avium*	IFO 3082	100
Serratia marcescens	IAM 1021	>100	*Mycobacterium smegmatis*	IFO 3083	100
Micrococcus lysodeikticus	IAM 1313	50	*Agrobacterium tumefaciens*	IAM 1037	>100
Micrococcus varians	IAM 1057	100	*Erwinia aroidae*	IAM 1068	>100
Staphylococcus aureus	IAM 1011	50	*Xanthomonas oryzae*	IAM 1657	>100
Staphylococcus aureus	IAM 1058	100	*Candida japonica*	IAM 4185	30
Sarcina lutea	IAM 1099	50	*Candida albicans*	IAM 4888	30
Brevibacterium ammoniagenes	IAM 1041	40	*Candida tropicalis*	IAM 4924	30
Corynebacterium equi	IAM 1038	20	*Saccharomyces fermentati*	IAM 4771	50
Microbacterium flavum	IAM 1642	25	*Saccharomyces cerevisiae*	IAM 4125	30
Microbacterium lacticum	IAM 1040	100	*Hansenula schneggi*	IAM 4269	30
Bacillus subtilis	IAM 1523	0.1	*Rhodotorula glutinis*	IAM 4642	30
Bacillus subtilis	IFO 3022	0.5	*Piricularia oryzae**		1

* Determined by paper disc method.

same as that against *Bacillus subtilis*.

Tunicamycin induces various morphological changes among sensitive microorganisms without inhibition of protein and nucleic acids syntheses as will be shown in another paper of this series (in preparation).

IV. Comparion with Already Known Antibiotics

The physical and chemical properties of tunicamycin show some resemblance to antibiotics such as mycospocidin[4], moenomycin[5], prasinomycins[6], macarbomycin[7] and diumycins[8]. But the above antibiotics, except mycospocidin, have phosphorus in their molecules, but tunicamycin does not. In addition, the molecular weight of tunicamycin was less than one-half of these antibiotics. Mycospocidin and tunicamycin have very similar melting points, elementary analyses, UV and IR spectra. Thin-layer chromatograms on cellulose, silicic acid and alumina of mycospocidin and tunicamycin are presented in Fig. 6. Differences between the two antibiotics were detected in some adsorbant-solvent systems. Mycospocidin and tunicamycin were found to be active on NDV to similar extents. Physical and chemical characteristics of the two antibiotics are summarized in Table 4 for comparison. Some differences are detected between the two, such as diazo reaction and presence of glycine or glucosamine in the hydrolysates.

Table 4. Summarized characteristics of mycospocidin and tunicamycin

	Mycospocidin	Tunicamycin
Producing organism	*Streptomyces bobiliae*	*Streptomyces lysosuperificus*
Ultraviolet absorption	215 mμ ($E^{1\%}_{1cm}$ 215) and 257~258 mμ ($E^{1\%}_{1cm}$ 89)	205 mμ ($E^{1\%}_{1cm}$ 230) and 260 mμ ($E^{1\%}_{1cm}$ 110)
Elementary analysis	C 54.26, H 7.57, N 6.43	C 52.59, H 7.67, N 6.25
Melting point	233~234°C (decomposition)	234~235°C (decomposition)
Color reaction	Diazo(+)	Diazo(−)
Products of hydrolysis with 6 N HCl	Glycine	Glucosamine (Glycine was not detected)
Minimum inhibitory concentrations against;		
Piricularia oryzae	25 mcg/ml	1 mcg/ml
NDV	1.5 mcg/ml	0.5 mcg/ml

The physical, chemical and biological characteristics of mycospocidin were reproduced from ref. 4, except the anti-NDV activity which was determined at the same time in our assay system.

Aknowledgement

The authors wish to express their sincere thanks to Meiji Seika Kaisha Ltd., for tank fermentations of tunicamycin and support in the characterization of the antibiotic. We are also grateful to Mr. K. AIZAWA and his co-workers of this Department for the measurement of IR, UV and NMR spectra and elementary analysis. The kind gift of mycospocidin by Prof. H. YONEHARA of the Institute of Applied Microbiology, the University of Tokyo is greatly appreciated.

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