VIRIDENOMYCIN,* A NEW ANTIBIOTIC

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Viridenomycin, a new crystalline antibiotic, was isolated from the culture broth of *Streptomyces viridochromogenes* strain No. T-24146. Viridenomycin is a weakly acidic and lipophilic substance, which exhibits a strong absorption maximum at 310 nm and shows strong activity against *Trichomonas vaginalis* and gram-positive bacteria.

A novel antibiotic, viridenomycin, has been obtained from the culture broth of a streptomycete (strain No. T-24146) isolated from a soil sample collected in Kyoto. The present paper shows that viridenomycin has strong activity against *Bacillus subtilis* and *Trichomonas vaginalis*, and is characterized by its strong absorption maximum at 310 nm in the ultraviolet spectrum. The producing organism is identified as a strain of *Streptomyces viridochromogenes* WAKSMAN and HENRICI, 1948[1].

Streptomyces sp. No. T-24146

Morphological properties of strain No. T-24146 are summarized as follows: The aerial mycelium branches monopodially and forms spore chains which belong to "Section Spira" according to PRIDHAM, *et al.*[2] (Plate 1). The spores are ellipsoidal to cylindrical (0.6~0.8×0.7~1.2 μ) with spiny surface (Plate 2).

Plate 1. Photomicrograph of sporophores of *S. viridochromogenes* strain No. T-24146 (×1,000×1/1.5)

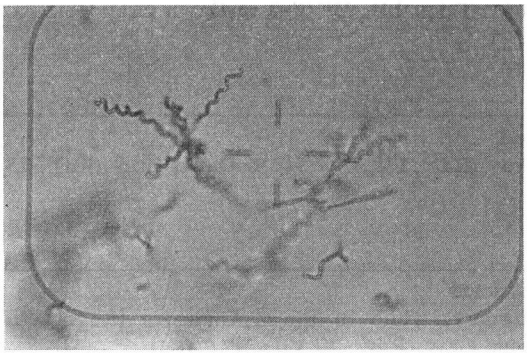
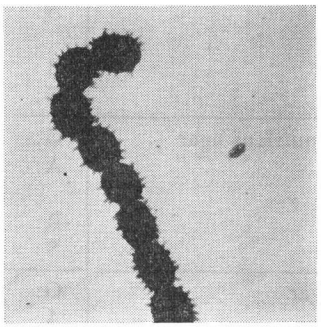


Plate 2. Electronmicrograph of spores of *S. viridochromogenes* strain No. T-24146 (×10,000×1/1.5)



Cultural and physiological properties of strain No. T-24146 are shown in Tables 1 and 2. The strain was determined to belong to the "Blue Series" of TRESNER and BACKUS[3] from the

* This antibiotic had been reported as viridomycin at the 186th meeting of Japan Antibiotics Research Association, but its name has been changed to viridenomycin.

Table 1. Cultural characteristics of *S. viridochromogenes* strain No. T-24146

Medium	Characteristics
CZAPEK's agar	G*: moderate, spreading A: moderate, powdery, white to Bluish Glaucous (Rdg., XLII, 37‴-f) or Court Gray (Rdg., XLVII, 29″ ″-f) R: Dark Bluish Glaucous (Rdg., XLII, 37″ ′-b) to yellowish gray S: none
Glucose CZAPEK's agar	G: good, wrinkled A: good, white to Green Glaucous Blue (Rdg., XLII, 41″ ′-b) R: dark greenish black S: brown
Glycerol CZAPEK's agar	G: moderate, wrinkled A: poor, powdery, white to Pale Mouse Gray (Rdg., LI, 15″ ″-d) R: dark grayish brown S: pale brown
Glucose asparagine agar	G: moderate A: moderate, powdery, white to Light Celandine Green (Rdg., XLVII, 33″ ″-b) R: brownish green S: brownish green
Calcium malate agar	G: moderate A: moderate, powdery, Dawn Gray (Rdg., LII, 35″ ‴-d) to Pale Olive Gray (Rdg., LI, 23″ ‴-f) R: Light Olive Gray (Rdg., LI, 23″ ‴-d) to Deep Olive Gray (Rdg., LI, 23″ ‴) S: pale greenish brown
Inorganic salts starch agar (ISP)	G: moderate A: moderate, powdery, Green Glaucous Blue (Rdg., XLII, 41″ ′-b) R: ivory S: pale yellowish brown
Nutrient broth	G: moderate, surface A: white to pale brown S: brown
Glycerol nutrient agar	G: good, wrinkled A: good, Peal Gray (Rdg., LII, 35″ ‴-f) to Dawn Gray (Rdg., LII, 35″ ‴-d) R: blackish brown S: dark brown
Glucose nutrient agar	G: good, wrinkled A: good, Peal Gray (Rdg., LII, 35″ ‴-f) to Dawn Gray (Rdg., LII, 35″ ‴-d) R: dark brown S: dark brown
Starch agar	G: moderate, restricted A: moderate, powdery, white to Bluish Glaucous (Rdg., XLII, 37″ ′-f) or Court Gray (Rdg., XLVII, 29″ ″-f) R: Dark Olive Buff (Rdg., XL, 21″ ′) to Dark Ivory Green (Rdg., XLVII, 25″ ″-k) S: none
Yeast extract agar	G: good, wrinkled A: abundant, Lilac Gray (Rdg., LII, 59″ ‴-f) to Hathi Gray (Rdg.,

Medium	Characteristics
Yeast extract agar	LII, 35″″-b) R: Olive Brown (Rdg., XL, 17″″-k) S: brown
Yeast extract malt extract agar (ISP)	G: good, wrinkled A: abundant, Green Glaucous Blue (Rdg., XLII, 41″″-b) R: yellowish brown S: amber
Oatmeal agar (ISP)	G: moderate A: moderate, powdery, Green Glaucous Blue (Rdg., XLII, 41″″-b) R: dark green S: none
Tyrosine agar	G: poor A: poor, white to Greenish Glaucous Blue (Rdg., XLII, 41″″-b) R: colorless to Ecru Drab (Rdg., XLVII, 13″″-d) S: none
Peptone agar	G: moderate, spreading A: moderate, powdery, Dawn Brown (Rdg., LII, 35″″-d) R: brown to dark brown S: pale brown
Starch ammonium agar	G: poor A: poor, white to Greenish Glaucous Blue (Rdg., XLII, 41″″-b) R: colorless S: none
Egg (cultivated at 37°C)	G: moderate, spreading A: moderate, powdery, white S: dark brown
Potato	G: moderate A: poor to moderate, white S: blakish brown
Carrot	G: none
Litmus milk (cultivated at 37°C)	G: moderate, surface, ring formed A: none S: pale brown
LOEFFLER's serum (cultivated at 37°C)	G: moderate, wrinkled A: moderate, white S: brown
Gelatin (cultivated at 24°C)	G: poor A: none S: brown
Cellulose	G: poor A: poor, greenish gray S: none

* G: Growth, A: Aerial mycelium, R: Reverse, S: Soluble pigment, Rdg.: RIDGWAY's "Color Standards and Color Nomenclature" Ref. 4).

fact that the color of aerial mycelium is light gray to light grayish blue. The vegetative mycelium (reverse side color of colony) is brown to brownish green or "Dark Bluish Glaucous" according to RIDGWAY's color standard.[4] Soluble pigment produced is brown to brownish

Table 2. Physiological properties of *S. viridochromogenes* strain No. T-24146

Temperature	growth occurs at 20~45°C better growth at 30~37°C
pH range	growth occurs at pH 4~10 optimum range at pH 7~8
Gelatin	liquefaction; weak to moderate
Litmus milk	peptonization without coagulation
Starch	hydrolysis; diameter of hydrolyzed zone/diameter of colony=40 mm/23 mm
Chromogenicity reaction	positive
Nitrate reduction	positive in peptone solution and CZAPEK's solution
Cellulose decomp.	negative
Liquefact. of serum	positive (weak)
Products	viridenomycin

Table 3. Utilization of carbon sources by *S. viridochromogenes* strain No. T-24146

Carbon sources	Growth	Carbon sources	Growth
Erythritol	+	Sucrose	++
Adonitol	+	Lactose	+++
D-Sorbitol	+	Raffinose	+++
i-Inositol	+++	Trehalose	+++
D-Mannitol	+++	Salicin	++~+++
Dulcitol	+	Esculin	+
D-Xylose	+++	Inulin	+
L-Arabinose	+	Na-succinate	++
L-Sorbose	±	Na-citrate	++
D-Galactose	+++	Na-acetate	+
D-Glucose	+++	D-Mannose	+++
D-Fructose	+++	Glycerol	+++
Rhamnose	+++	Starch	+++
Melibiose	+++	Control	±
Maltose	+++		

+++: abundant growth ++: good growth +: moderate growth
±: poor growth −: no growth

green on synthetic media and brown to dark brown on organic media. Consequently, strain No. T-24146 belongs to a chromogenic type.

Utilization of various carbon sources by the strain was investigated by PRIDHAM's method[5]. The strain assimilates more or less all carbon sources examined except L-sorbose (Table 3).

When the above-mentioned characteristics of strain No. T-24146 are compared with those of *Streptomyces* species described by WAKSMAN[6], PRIDHAM[2], HÜTTER[7] and others, strain No. T-24146 is similar to *S. viridochromogenes* WAKSMAN and HENRICI, 1948[1], *S. cyaneus* WAKSMAN, 1953[8], *S. chartreusis* CALHOUN and JOHNSON, 1956[3], *S. coeruleofuscus* GAUZE, *et al.*, 1957[9], *S. coeruleorubidus* GAUZE, *et al.*, 1957[9], *S. bellus* MARGALITH and BERETTA, 1959[10], *S. curacoi* CATALDI, 1962[11], *S. glaucescens* GAUZE, *et al.*, 1957[9] and *S. coerulescens* GAUZE, *et al.*, 1957[9].

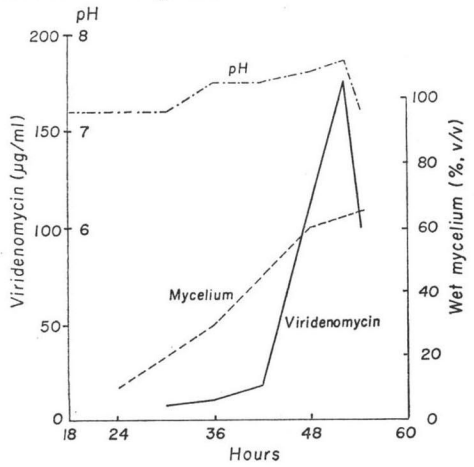
In the color of reverse side of the colony on various media and in its physiological characteristics, strain No. T-24146 is distinguished from *S. cyaneus*, *S. chartreusis*, *S. coeruleofuscus*, *S. coeruleorubidus* and *S. bellus*. *S. curacoi*, *S. glaucescens* and *S. coerulescens* can be scarcely distinguished taxonomically from *S. chartreusis* except for the antibiotics produced, and hence they are differentiated from strain No. T-24146.

Judging from the detailed comparisons, it was recognized that strain No. T-24146 is closely related to *S. viridochromogenes* WAKSMAN and HENRICI, 1948 in morphological and physiological characteristics. Strain No. T-24146 was subsequently compared with *S. viridochromogenes* IFO 12337 (ATCC 13729) under the same cultural conditions, and no noticeable difference was found between them in cultural, morphological and physiological properties. These facts led to the conclusion that strain No. T-24146 belongs to *S. viridochromogenes* WAKSMAN and HENRICI, 1948. The strain has been deposited in the Institute for Fermentation, Osaka and assigned accession number IFO 13188.

Antibiotic Production

Stock cultures of *S. viridochromogenes* strain No. T-24146 grown on glucose asparagine agar slants for 5~7 days at 28°C were maintained at 4~6°C. In the tank fermentation, spores from these stock cultures were used to inoculate a 2-liter SAKAGUCHI flask containing 500 ml of seed culture medium consisting of 2% glucose, 3% soluble starch, 1% soybean flour, 1% corn steep liquor, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$ (initial pH: 7.0). After incubation for 48 hours at 28°C on a reciprocal shaker, the culture was transferred to a 200-liter tank fermentor containing 100 liters of the seed culture medium described above and incubated at 28°C for 48 hours with stirring at 200 rpm and aerating at 100 liters/min. Then the resulting seed culture (100 liters) was transferred to a 2,000-liter stainless steel tank containing 1,000 liters of the main culture medium. The main culture medium was composed of 2% glucose, 1% glycerol, 2% soluble starch, 1% soybean flour, 1% corn steep liquor, 1% cotton seed meal, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$ (initial pH: 7.0). The fermentation was carried out with stirring at 120 rpm for 52 hours at 28°C. A typical fermentation process is shown in Fig. 1.

Fig. 1. Time course of viridenomycin production by *Streptomyces viridochromogenes* strain No. T-24146. Test organism for potency assay: *Trichomonas vaginalis*



During the fermentation and isolation process, the antiprotozoal activity was determined by broth dilution method using *Trichomonas vaginalis* No. 4F, and the antibacterial activity was assayed by the agar dilution method using *Bacillus subtilis* PCI 219.

Isolation and Purification

Most of the antibiotic contained in the culture broth was found to be adsorbed on the mycelial cake, when the broth was filtered with filter-aid after acidification. Consequently, the

fermentation broth (900 liters) was acidified to pH 2 with 4 N HCl and filtered with the aid of 2% Hyflo-Super Cel. The mycelial cake (200 kg) obtained was stirred for 1 hour with ethyl acetate containing 2% oxalic acid. The ethyl acetate layer was washed with 5% NH_4OH and water, and then concentrated *in vacuo* to obtain an oily residue. To the residue were added 20 liters of *n*-hexane and the mixture was allowed to stand in the refrigerator overnight to yield a precipitate. The precipitate (500 g) was separated by filtration and dissolved in a mixture of tetrahydrofuran and ethyl acetate (1 : 3) (3 liters). The solution was then charged on a column packed with alumina (5 kg, Merck; activity II-III) which was previously washed with ethyl acetate containing 2% oxalic acid, and the column was developed with etheyl acetate. The active fractions of eluate were collected and evaporated *in vacuo* to obtain a crude yellow powder (300 g). The powder was purified by dissolving in tetrahydrofuran (1 liter) and subsequent precipitation by addition of methanol (2 liters). A pale yellow powder (200 g) thus obtained was crystallized from tetrahydrofuran-methanol and recrystallized from chloroform-methanol to give viridenomycin as colorless fine plates.

Physical and Chemical Properties

Viridenomycin is obtained as weakly acidic and lipophilic crystals. Its physical and chemical properties are summarized in Table 4. Characteristic physical properties of viridenomycin are its strong ultraviolet absorption, $E^{1\%}_{1cm}$ 1,879, at 310 nm in methanol (Fig. 2) and strong dextrorotation, $[\alpha]^{32}_{D}+893°$ (*c* 0.5, chloroform). Fig. 3 shows the infrared absorption spectrum of viridenomycin in KBr disk.

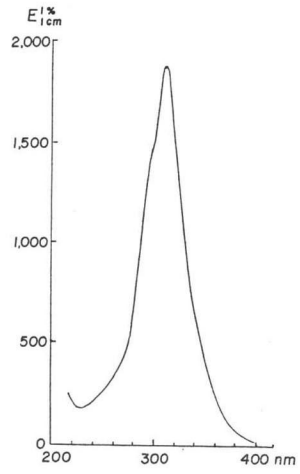
Table 4. Physical and chemical properties of viridenomycin

Appearance	Colorless fine plates
Melting point	168~170°C (dec.)
Elemental analysis	C 72.09, 71.70; H 7.01, 6.91; N 2.42, 2.37, no halogen, phosphorus
Molecular weight	566 (vapor pressure osmometry: solvent ethyl acetate)
Molecular formula	$C_{34-36}H_{35-41}NO_{6-7}$
Optical rotation	$[\alpha]^{32}_{D}+893°$ (*c* 0.5 in chloroform)
Ultraviolet absorption	λ^{MeOH}_{max} 310 nm ($E^{1\%}_{1cm}$ 1,879)
pKa' value	8.8 [dimethyl sulfoxide−H_2O (77 : 22)]
Solubility	Easily soluble in dimethyl sulfoxide, tetrahydrofuran, pyridine Soluble in chloroform, ethyl acetate, methanol Insoluble in water, ether, benzene, petroleum ether, *n*-hexane
Color reaction	Positive: $KMnO_4$ (decoloration), ferric chloride, DRAGENDORFF Negative: ninhydrin, EHRLICH, anthrone, ELSON-MORGAN, WOOD
Rf values on silica gel TLC (Tokyo Kasei: Spot Film)	$CHCl_3$-MeOH (9 : 1) 0.2 Benzene-$CHCl_3$-MeOH (70 : 28 : 2) 0.2 (pre-treated TL with oxalic acid)
Stability (MeOH solution)	Relatively stable in the dark, unstable to UV light

Biological Properties

The antimicrobial spectrum against bacteria and fungi was determined by the conventional serial agar dilution method, and the growth inhibition assay with protozoa was performed by the broth dilution method using a medium (SYS medium) consisting of 0.2% L-cysteine, 0.5%

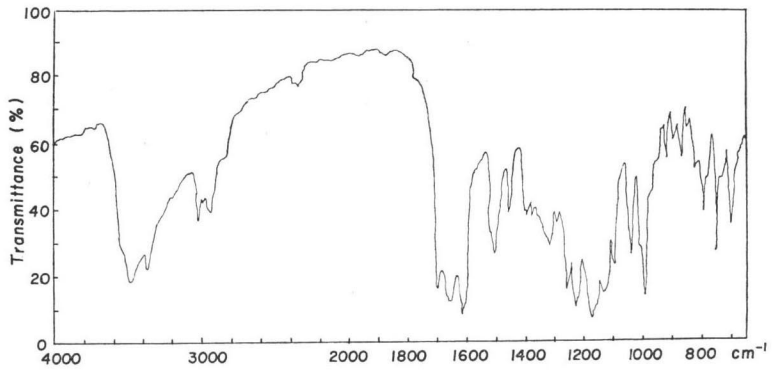
Fig. 2 Ultraviolet absorption spectrum of viridenomycin (in methanol)



NaCl, 1.0 % glucose, 1.0 % yeast extract, 2 % peptone and 10 % horse serum (pH 5.6). Table 5 shows the antimicrobial spectrum of viridenomycin.

Viridenomycin shows strong activity against *Trichomonas vaginalis* No. 4F; the minimum inhibitory concentration is 0.06 mcg/ml. Viridenomycin is also active against gram-positive bacteria, especially against *Bacillus subtilis*, slightly active against mycobacteria and fungi, but inactive against yeast and gram-negative bacteria. Viridenomycin also has an inhibitory activity on the growth of pleuropneumonia-like organisms (PPLO). For example, the minimum inhibitory concentration against *Mycoplasma gallisepticum* in the standard broth dilution test is 0.01 mcg/ml.

Fig. 3. Infrared absorption spectrum of viridenomycin in KBr disk



The acute toxicity (LD_{50}) in mice was about 250, 500~1,000 and 2,000~4,000 mg/kg, when administered by intraperitoneal, subcutaneous and oral routes, respectively.

Comparison with Other Known Antibiotics

Comparison of viridenomycin with other known antibiotics which have ultraviolet absorption at 300~330 nm is shown in Table 6. The ultraviolet absorbance ($E^{1\%}_{1cm}$) at around 310 nm and the optical rotation ($[\alpha]_D$) and the antiprotozoal activity of viridenomycin are outstanding. From these physicochemical and biological properties, viridenomycin is considered to be a novel antibiotic.

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Table 5. Antimicrobial activity of viridenomycin

Test organisms	MIC (mcg/ml)	Medium*
Escherichia coli IFO 3044	>100	I
Proteus vulgaris IFO 3045	>100	I
Pseudomonas aeruginosa IFO 3080	>100	I
Staphylococcus aureus FDA 209P	0.5~1.0	I
Bacillus subtilis PCI 219	0.05	I
Bacillus cereus IFO 3466	5~ 10	I
Bacillus brevis IFO 3331	2~ 5	I
Sarcina lutea IFO 3232	2~ 5	I
Micrococcus flavus IFO 3242	5~ 10	I
Mycobacterium sp. Takeo IFO 3153	50~100	II
Mycobacterium smegmatis IFO 3083	50	II
Mycobacterium phlei IFO 3158	20	II
Mycobacterium sp. ATCC 607	50~100	II
Mycobacterium bovis BCG	25	III
Penicillium chrysogenum IFO 4626	20~ 50	IV
Saccharomyces cerevisiae IFO 0209	>100	IV
Candida albicans IFO 0583	50	IV
Aspergillus niger IFO 4066	100	IV
Trichophyton mentagrophytes IFO 5809	10~ 20	IV
Trichomonas vaginalis No. 4F	0.06	V

* Medium used. I: bouillon agar, II: glycerol-bouillon agar, III: KIRCHNER-albumin, IV: glucose-bouillon agar, V: SYS medium.

Table 6. Comparison of viridenomycin with the known antibiotics with ultraviolet absorptions at 300~330 nm

	Viridenomycin	Denamycin[12]	Akitamycin[13]	Peresimycin[14]	Ikarugamycin[15]	Thaimycin B[16]	Variotin[17]
Melting point (°C)	168~170 (dec.)	226~228	180 (dec.)	145	252~255 (dec.)	260 (dec.)	oil
$[\alpha]_D$	+893°	+41°	+88°	+695°	+360°	+45°	+5.68°
Elementary analysis C	72.09	68.26	57.26	44.03	72.35	67.26	69.74
H	7.01	8.71	7.68	8.31	8.09	7.97	8.66
N	2.42	0	1.64	8.56	5.87	5.98	4.72
UV absorption							
λ_{max}^{MeOH}	310	311	291, 304, 319	309	220, 325	238, 321	320
$E_{1cm}^{1\%}$	1879	520	600, 700, 640	278	600, 418	449, 255	1198
Antiprotozoal activity MIC (mcg/ml)	0.06	not described	12.5	not described	0.3~1.25	0.5	not described

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