AM-2604 A, A NEW ANTIVIRAL ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES*

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A novel antibiotic, AM-2604 A was isolated from the fermentation broth of *Streptomyces* sp. AM-2604 by solvent extraction and silica gel column chromatography. The antibiotic is pale yellow needles possessing UV absorption maxima at 280 nm and 330 nm (sh). The antibiotic possesses weak activity against fungi and trichomonad and potent inhibitory activity against various RNA and DNA viruses *in vitro*.

In the course of our screening program for new antiviral substances from actinomycetes, we previously found a new antiviral substance, virantmycin.[1,2] Furthermore it was found that *Streptomyces* sp. AM-2604 isolated from a soil sample collected at Tachikawa City, produces a novel antibiotic, AM-2604 A, possessing potent activity against both RNA and DNA viruses *in vitro*. This paper deals with the taxonomy of the producing strain, the fermentation, the isolation and the physicochemical and biological properties of the antibiotic.

Taxonomy

The producing microorganism, strain AM-2604, was isolated from a soil sample collected in Tachikawa City, Tokyo, Japan. Taxonomic studies of the strain were carried out by the method of SHIRLING & GOTTLIEB[3] and WAKSMAN.[4] The color of the culture was determined with reference to the Color Harmony Manual.[5] The morphology of spore chains and spores was observed with electron microscopy.

Fig. 1 shows the electron micrograph of the conidia of strain AM-2604, cultured on oatmeal agar at 27°C for 2 weeks. The sporophore were spiral or loop type, and contained more than ten spores per chain. The spores were oval in shape, warty on the surface, and $0.8 \times 1.3 \sim 1.5$ μm in diameter. Sclerotic granucles, sporangia and zoospores were not observed.

Table 1 shows the characteristics of cultures incubated at 27°C for 14 days. The vegetative mycelia without septa developed abundantly in every culture. The aerial mycelia developed abundantly on some media, were white or gray, and velvety or cottony on the surface. No soluble

Fig. 1. Electron micrograph of the conidia of strain AM-2604 (bar presents 1 μm).

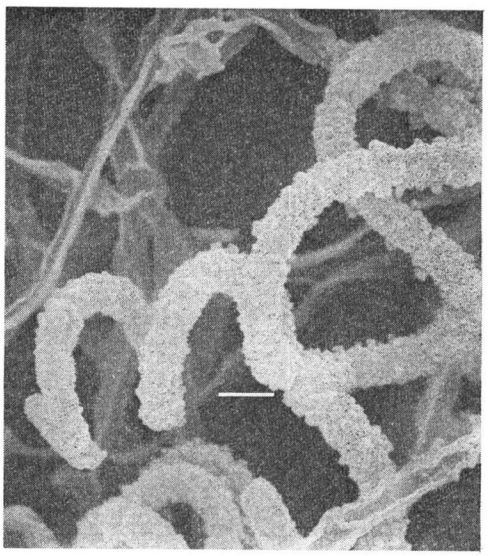


Table 1. Cultural characteristics of strain AM-2604.

	Growth	Reverse	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP)*	Good, raised, bamboo (2gc)	Gold (21c)	Moderate, cottony, white ashes (a 5fe)	None
Oatmeal agar (ISP)*	Good, penetrated, light ivory (2ca)	Light ivory (2ca)	Good, velvety, pussywillow gray (5dc)	None
Inorganic salts-starch agar (ISP)*	Good, light ivory (2ca)	Light ivory (2ca)	Good, velvety, ashes (5fe)	None
Glycerol-asparagine agar (ISP)*	Good, raised, pearl (2ba)	Light ivory (2ca)	Moderate, velvety, oyster white (b)	None
Glucose-asparagine agar	Good, raised, light ivory (2ca)	Light ivory (2ca)	Moderate, velvety, oyster white (b)	None
Peptone-yeast extract iron agar (ISP)*	Poor, light wheat (2ea)	Light wheat (2ea)	None	None
Tyrosine agar (ISP)*	Thin, yellow tint (1ba)	Yellow tint (1ba)	None	None
Sucrose-nitrate agar**	Good, light wheat (2ea)	Light wheat (2ea)	Moderate, velvety, white (a)	None
Glucose-nitrate agar**	Moderate, light wheat (2ea)	Light wheat (2ea)	None	None
Glycerol-calcium-malate agar**	Good, penetrated, parchment (1cb)	Parchment (1cb)	Poor, white (a)	None
Glucose-peptone agar**	Good, light wheat (2ea)	Light wheat (2ea)	None	None
Nutrient agar*	Poor, light ivory (2ca)	Light ivory (2ca)	None	None

* Medium recommended by the International Streptomyces Project.
** Medium recommended by S. A. WAKSMAN[2].

Table 2. Physiological properties of strain AM-2604.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	+
Liquefaction of gelatin (20°C)	+
Hydrolysis of starch	+
Coagulation of milk (37°C)	+
Peptonization of milk (37°C)	+
Cellulolytic activity	−
Temperature range for growth	15~40°C

Table 3. Utilization of carbon sources by strain AM-2604.

D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
D-Fructose	+
L-Rhamnose	+
i-Inositol	+
Sucrose	+

pigments were observed in every culture.

Tables 2 and 3 show the physiological properties and the utilization of carbon sources, respectively. No melanin formation was observed. Every carbon source tested was utilized for growth by the strain. The diaminopimelic acid was LL-type in cell wall of the mycelia, arabinose and galactose were not contained.

From the properties described above, strain AM-2604 is assigned to the white or gray series of the genus *Streptomyces* in PRIDHAM & TRESNER's classification scheme.[6]

The strain has been deposited with the Fermentation Research Institute, Agency of Industrial

Science and Technology, Japan, as *Streptomyces* sp. AM-2604 under the accession number FERM-P 6278.

Fermentation

A loopful of the spores and mycelia on an agar medium slant was inoculated into a seed medium (100 ml) consisting of 1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% peptone and 0.4% $CaCO_3$ (pH 7.0) in a Sakaguchi flask (500 ml), and incubated at 27°C for 48 hours on reciprocal shaker, to give the seed culture. The seed culture (700 ml) were transferred into a production medium (70 liters) consisting of 2% dextrin, 0.2% glucose, 1.5% soybean meal, 0.3% yeast extract and 0.3% $CaCO_3$ (pH 7.0) in jar fermentor (100 liters). Fermentation was carried out at 27°C for 67 hours with aeration (35 liters/minute) and agitation (200 rpm).

The antibiotic production was assayed by a plaque reduction method[1] with vesicular stomatitis virus (VSV) and detected by thin-layer chromatography on silica gel.

The production of the antibiotic, AM-2604 A in the fermentation broth reached to a maximum at about 42~52 hours.

Isolation and Purification

The fermentation broth (70 liters) was centrifuged with a Sharpless type centrifuge. The mycelial cake obtained was suspended in 60% aqueous acetone (10 liters) with stirring for 20 minutes. The filtrate, prepared by filtration, was concentrated *in vacuo* to water-rich solution (5.2 liters), and it was extracted with ethyl acetate (10 liters). The ethyl acetate extract was concentrated *in vacuo*, to give dark-brown paste (1.2 g). The residue (1.1 g) was chromatographed over silica gel (Kieselgel 60, 25 g) column with benzene - acetone (5: 1 to 3: 1, v/v) as eluents. The active eluates were combined, and concentrated *in vacuo* to 10 ml, to give yellow precipitate. Yellow powder was separated from the solution by filtration. The powder was crystallized from acetone to give pale yellow needles (680 mg).

Physicochemical Properties

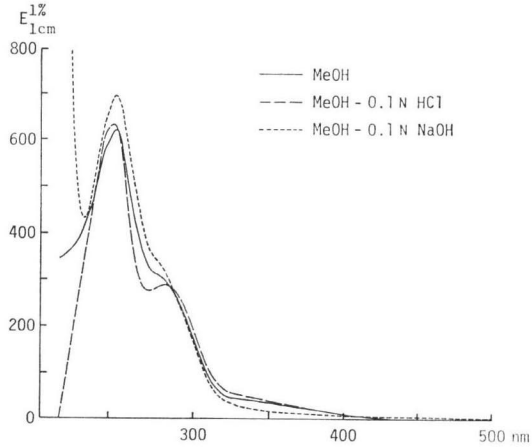
The antibiotic is lipophilic pale-yellow needles, mp 204~205°C and $[\alpha]_D^{27.5}+240°$ (*c* 0.02, MeOH). The antibiotic did not give definite values in elemental analysis (C 64~65%, H 7~8%, N 1.5~1.6%) and molecular ion peak in the EI and FD-mass spectra. Therefore, the accurate molecular formula could not be obtained, the molecular formula, $C_{47\sim48}H_{69\sim74}NO_{14\sim15}$ for AM-2604 A was estimated on the basis of ^{13}C NMR and 400 MHz ^1H NMR spectral analyses. The UV spectrum exhibits a maxi-

Table 4. Rf values of AM-2604 A on thin-layer chromatography.

Silica gel 60 F_{254} plates (0.2 mm thick, Merck) were used.

Solvent system	Rf value
$CHCl_3$ - MeOH (9: 1, v/v)	0.54
$CHCl_3$ - MeOH (4: 1, v/v)	0.73
Benzene - acetone (1: 1, v/v)	0.58
Benzene - MeOH (2: 1, v/v)	0.70
AcOEt	0.36
EtOH - H_2O (4: 1, v/v)	0.81

Fig. 2. UV spectra of AM-2604 A.



mum peak at 248 nm and shoulder peaks at 280 nm and 330 nm in methanol, as shown in Fig. 2. The IR spectrum (KBr) shows characteristic absorption bands at 3475, 3380, 1720, 1690, 1625, 1550, 1445, 1360, 1250, 1165, 1105 and 965 cm^{-1}, as shown in Fig. 3. The ^1H NMR spectrum exhibited the presence of olefinic protons, two methoxy groups, and methyl and methylene groups, as shown in Fig. 4. The antibiotic gives positive reaction to ferric chloride, potassium permanganate, anisaldehyde-sulfuric acid and *p*-anisidine-hydrochloric acid, but negative to DRAGENDORFF and ninhydrin. The antibiotic is soluble in

Fig. 3. IR spectrum of AM-2604 A in KBr.

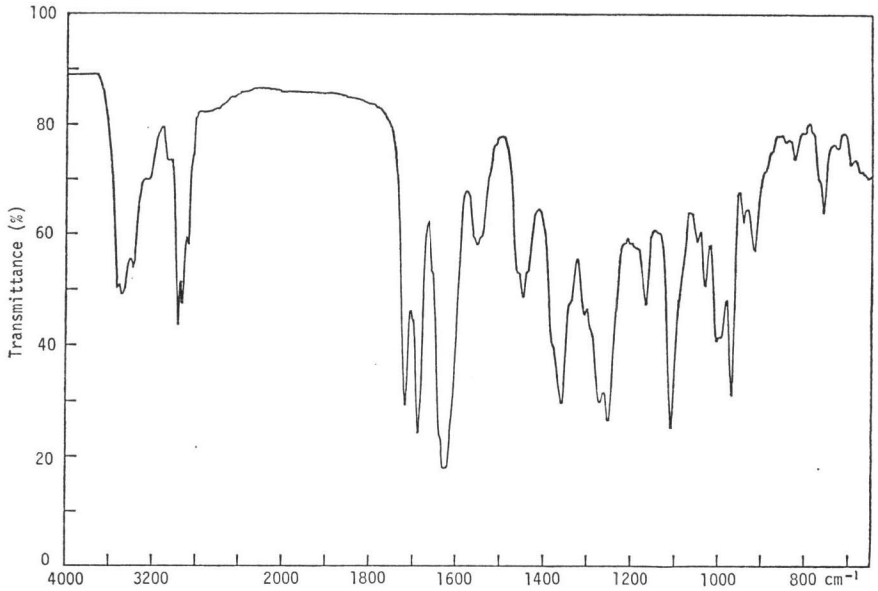
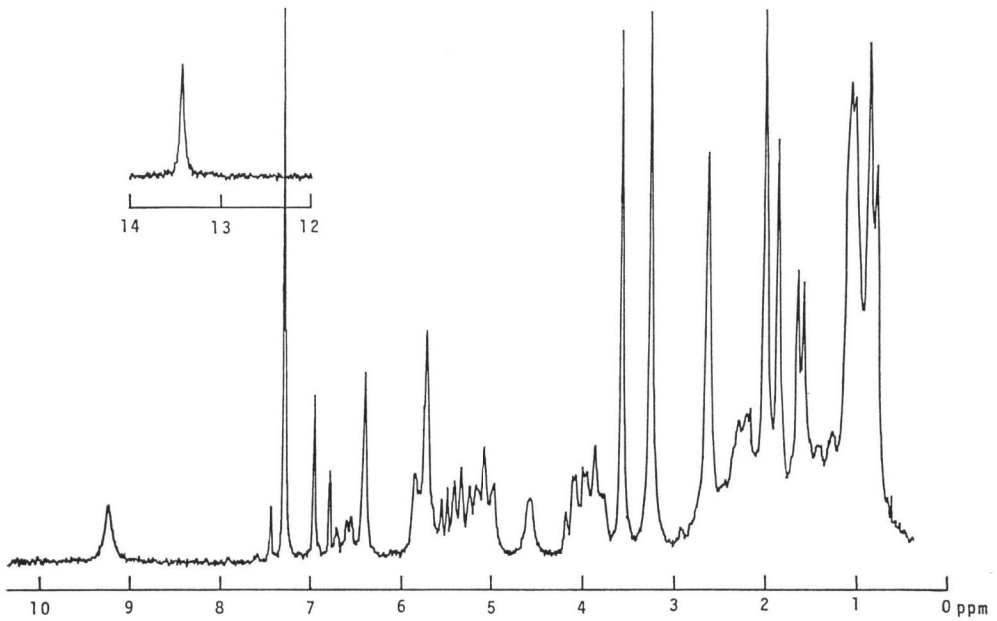


Fig. 4. ^1H NMR spectrum of AM-2604 A (100 MHz, in $CDCl_3$).



chloroform, ethyl acetate and acetone, slightly soluble in methanol and ethanol, and insoluble in water and *n*-hexane. The Rf values on silica gel thin-layer chromatography of AM-2604 A are shown in Table 4.

Biological Properties

Both primary chick embryonic (CE) cells and established cell line RK-13 cells, cultivated in minimum essential medium (MEM) supplemented with 10% calf serum, were used for assay of antiviral activity. The following eight viruses were used as challenge viruses: New Jersey strain of vesicular stomatitis virus (VSV), Egypt Ar 339 strain of Sindibis virus (SbV), McMillan strain of Western equine encephalitis virus (WEE), Miyadera strain of Newcastle disease virus (NDV), DIE strain of vaccinia virus (Vac-DIE), IHD strain of vaccinia virus (Vac-IHD), HF strain of herpes simplex virus type 1 (HSV-1), UW strain of herpes simplex virus type 2 (HSV-2). Antiviral activity with the guidance of plaque reduction was carried out according to the methods described previously.[1)]

Table 5 shows the antiviral activity of the antibiotic against several RNA and DNA viruses with CE cells. The antibiotic diminished 50% of plaque formation caused by both RNA and DNA viruses at very low concentrations ($3\times10^{-4}\sim5\times10^{-5}$ μg/ml). At the same time morphological cytotoxicity was observed at 1×10^{-2} μg/ml with light microscopy. Furthermore, at the concentration 4×10^{-2} μg/ml the antibiotic inhibited 50% of RK-13 cell growth after 48 hours incubation in MEM plus 10% calf serum, and at the concentration 5×10^{-4} μg/ml it inhibited 50% of plaque formation caused by VSV with RK-13 cells.

Table 6 shows the minimum inhibitory concentrations of the antibiotic against various microorganisms. The antibiotic is weakly active against fungi such as *Microsporum gypseum*. *Trichophyton interdigitale*, *Piricularia oryzae*, *Mucor racemosus* and *Monilinia fructicola*, and

Table 5. Antiviral activity of AM-2604 A against several RNA and DNA viruses.

Virus		ED_{50}* (μg/ml)
RNA virus	VSV	0.0003
	SbV	0.0005
	WEE	0.0003
	NDV	0.0003
DNA virus	Vac-DIE	0.0003
	Vac-IHD	0.0003
	HSV-1	0.0003
	HSV-2	0.0003

* 50% Effective dose, measured by plaque reduction method, described previously.[1)]

Table 6. Antimicrobial spectrum of AM-2604 A.

Organism	Medium* and method	MIC** (μg/ml)
Staphylococcus aureus ATCC 6538P	I	>100
Bacillus subtilis ATCC 6633	I	>100
Micrococcus luteus ATCC 9341	I	>100
Mycobacterium smegmatis ATCC 607	I	>100
Escherichia coli NIHJ	I	>100
Proteus vulgaris IFO 3167	I	>100
Xanthomonas oryzae	II	>50
Candida albicans	II	>100
Saccharomyces sake	II	100
Microsporum gypseum	II	50
Trichophyton interdigitale	II	50
Aspergillus niger	II	>100
Piricularia oryzae	II	12.5
Alternaria kikuchiana	II	>50
Mucor racemosus	II	50
Fusarium oxysporum	II	>100
Monilinia fructicola	II	12.5
Botrytis cinerea	II	>50
Trichomonas foetus	III	25
Trichomonas vaginalis	III	6.25

* I: Heart infusion agar, agar dilution method (37°C, 20 hours).
II: Potato glucose agar, agar dilution method (27°C, 48 hours).
III: Trichocel broth (BBL), broth dilution method (37°C, 48 hours).

** Minimum inhibitory concentration.

trichomonads such as *Trichomonas foetus* and *Trichomonas vaginalis*, but inactive against bacteria and yeasts.

Acute toxicity of the antibiotic was examined with mice, all of the mice were survived by intraperitoneal injection of 1 mg/kg, but all of the mice died at the injection of 10 mg/kg.

Discussion

Among known antibiotics, setamycin[7], SF-1540 A[8], SF-1540 M[9] and SF-1540 B[8] resemble the antibiotic AM-2604 A in ultraviolet absorption. Setamycin, SF-1540 A and SF-1540 M exhibit ultraviolet absorptions at 249 nm (maximum) and 280 nm (sh). SF-1540 B shows those at 247 nm and 277 nm. However AM-2604 A can be differentiated from SF-1540 B by the absence of nitrogen, and from setamycin, SF-1540 A and SF-1540 M by the large difference in melting points and the absence of activity against Gram-positive bacteria. Furthermore, AM-2604 A is differentiated from setamycin, SF-1540 A and SF-1540 M by Rf values of TLC on silica gel with benzene - acetone (3: 1): AM-2604 A, 0.15; setamycin, 0.22; SF-1540 A, 0.23. It has been reported that the Rf value of SF-1540 M is larger than that of SF-1540 A[9]. Thus, AM-2604 A is considered to be a new antibiotic.

No study about antiviral activities of the antibiotics described above has been reported. AM-2604 A is an interesting substance, for it possesses potent antiviral activities against both RNA and DNA viruses which differ from each other in their multiplication mechanisms.

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