A NEW ANTIVIRAL SUBSTANCE S-15-1, STREPTOTHRICIN GROUP ANTIBIOTIC

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In the screening for antiviral antibiotics produced by *Streptomyces* soil isolates, several strains were found to produce cytotoxic and/or antiviral antibiotics. One antiviral antibiotic designated S-15-1 was isolated from soil isolate *Streptomyces* S-15-1. The antibiotic S-15-1 shows noncytotoxic *in vitro* inhibitory activity against Newcastle disease virus, and it has also inhibitory activity against gram-positive, gram-negative bacteria as well as some yeasts.

In the course of an antiviral antibiotic screening program utilizing a paper-disc agar diffusion technique[1] involving Newcastle disease virus (NDV) MIYADERA strain[2] with a primary culture of chick embryo fibroblast (CEF), it was found that 62 strains of unidentified *Streptomyces* obtained from soil samples produced cytotoxic and/or antiviral antibiotics. One of the isolates, *Streptomyces* S-15-1, produced a novel water-soluble basic antibiotic. This noncytotoxic antibiotic, designated S-15-1, was active *in vitro* against NDV and active against gram-positive, gram-negative bacteria, and some strains of yeast.

This paper describes the screening for antiviral antibiotics produced by one isolate of *Streptomyces*, and also the production, isolation, purification, and physicochemical and biological properties of S-15-1.

Screening of the Antiviral Antibiotics

Approximately one thousand strains of *Streptomyces* obtained from soil samples were inoculated in each of two media and cultured at 26.5°C for 5 days with shaking. The media were as follows: medium A contained 3.0 % glycerol, 1.0 % peptone, 0.5 % $(NH_4)_2HPO_4$, 1.0 % K_2HPO_4, 0.5 % NaCl, 0.05 % $MgSO_4 \cdot 7H_2O$, and 0.001 % $FeSO_4 \cdot 7H_2O$ (pH 7.0); medium B contained 3.0 % starch, 1.0 % peptone, 0.4 % $NaNO_3$, 0.2 % K_2HPO_4, 0.1 % KCl, 0.1 % $MgSO_4 \cdot 7H_2O$, and 0.002 % $FeSO_4 \cdot 7H_2O$ (pH 7.0).

After cultivation an equal volume of acetone was added to the whole broth, and antiviral activity in supernatant of broth was examined by the method of HERRMANN and his coworkers, and at the same time antimicrobial activity was also determined. In all, 47 strains produced only the cytotoxic and/or antiviral antibiotics and 15 strains produced the cytotoxic and/or antiviral as well as antimicrobial activity. On the other hand, 126 strains of *Streptomyces* produced the antimicrobial antibiotics.

Table 1 indicates the strains which produced the cytotoxic and/or antiviral antibiotics. Cytotoxic activity was observed more frequently than antiviral activity.

Table 1. Antiviral and cytotoxic activity of the antibiotics produced by *Streptomyces* obtained from soil samples

Organisms	Medium	CTZ	PIZ	Organisms	Medium	CTZ	PIZ	Organisms	Medium	CTZ	PIZ	Organisms	Medium	CTZ	PIZ
S 1-2	B	19	—	S 6-3	B	22	—	S 12-6	B	12	—	S 16-22	B	13	—
S 2-2	B	18	—	S 6-4	B	12	—	S 12-11	B	12	—	S 16-32	B	—	16
S 2-3	B	18	—	S 6-5	A	11	—	S 12-18	B	20	—	S 16-33	B	9	11
S 2-4	B	—	12	S 7-1	B	—	8	S 12-21	B	12	—	S 17-1	B	17	—
S 2-8	B	10	—	S 8-10	B	26	—	S 13-2	B	8	—	S 17-3	B	—	11
S 2-10	B	—	18	S 8-11	B	25	—	S 13-22	B	—	12	S 17-5	B	10	—
S 2-12	B	15	—	S 8-12	B	10	—	S 13-23	B	—	10	S 17-7	B	8	—
S 2-14	B	20	—	S 9-9	B	24	—	S 14-4	B	16	18	S 18-2	B	22	—
S 2-16	A	—	7	S 9-10	B	23	—	S 16-11	B	—	10	S 19-4	B	7	—
S 2-18	B	11	17	S 10-4	B	18	—	S 16-12	B	20	—	S 19-6	B	18	—
S 4-4	B	19	—	S 10-6	A	—	10	S 16-13	B	14	—	S 19-31	B	—	15
S 5-4	B	—	14	S 10-11	B	10	14	S 16-21	B	10	—				

Monolayers of chick embryo fibroblast formed on Petri dish (90 mm in diameter) using the E.C. HERRMAN, Jr., *et al.* method were infected by NDV MIYADERA strain and overlayed with soft agar medium. Paper discs impregnated with samples (Toyo Roshi Co., 6 mm in diameter) were placed on the agar overlay and plates were incubated at 37°C. After 48-hour incubation, diameters of plaque-free protected zones (PIZ) and cytotoxic zones (CTZ) were measured and expressed in mm.

Table 2. Antiviral and cytotoxic activity and antimicrobial activity of the antibiotics produced by *Streptomyces* obtained from soil samples

Organisms	Midia	CTZ	PIZ	Growth inhibition zone			
				S. lutea	*B. subtilis*	*E. coli*	*C. albicans*
S 2-1	B	13	—	14.5	—	—	12.0
S 2-5	B	12	—	12.0	—	—	10.0
S 2-6	B	11	16	—	—	—	15.0
S 5-2	A	12	16	19.8	—	—	—
S 6-1	B	—	9	16.6	13.6	—	19.8
S 6-8	B	19	—	20.2	27.5	—	10.7
S 8-14	B	16	—	—	13.0	—	—
S 9-2	A	—	12	23.5	11.8	—	—
S 15-1	B	—	19	17.4	20.2	10.6	15.0
S 16-1	A, B	17	—	—	14.3	—	16.4
S 17-7	B	24	—	15.0	14.0	—	—
S 17-20	B	30	—	12.0	8.5	—	—
S 17-21	B	14	—	12.5	13.0	—	—
S 19-17	B	16	—	—	10.0	10.0	—
S 19-25	B	—	18	—	—	12.0	—

The NDV MIYADERA strain and CEF monolayer system was used. Diameters of plaque-free protected zone (PIZ) and cytotoxic zone (CTZ) were measured and expressed in mm. The paper disc used was 6 mm in diameter for broth antiviral and cytotoxic activity, and 8 mm in diameter for antimicrobial activity (Toyo Roshi Co.).

A few strains produced high concentrations of cytotoxic activity and small amounts of antiviral activity.

Table 2 shows the strains which produced cytotoxic and/or antiviral and antimicrobial antibiotics. *Streptomyces* isolated from soil samples grow very poor on medium A, and some strains produce lower concentrations of antibiotics in this medium (Tables 1 and 2).

Production, Isolation, and Purification of S-15-1

Strain S-15-1 was of particular interest because of produced noncytotoxic antiviral activity with very broad spectrum antimirobial activity. *Streptomyces* S-15-1 was grown in 20 liters of medium, consisting of 3.0 % starch, 1.0 % peptone, 0.4 % $NaNO_3$, 0.2 % K_2HPO_4, 0.1 % KCl, 0.1 % $MgSO_4 \cdot 7H_2O$, and 0.002 % $FeSO_4 \cdot 7H_2O$ (pH 7.0), sterilized at 120°C for 20 minutes in a 30-liter stainless steel jar fermentor. Each jar was inoculated with 5.0 % (v/v) of 48-hour-old seed culture grown in the same medium in shaker flasks at 26.5°C. The fermentation was carried out in a jar fermentor for 55 hours at 26.5°C, agitating 280 rpm, with 100 % aeration. The

final pH of the culture broth was 7.8. Fermented medium (40 liters) was adjusted to pH 6.0 with diluted hydrochloric acid, and filtered to remove cell debris. The culture filtrate was adjusted to pH 7.2 and stirred with active carbon (5%, v/v). After the filtration of carbon, the carbon cake was washed twice with water and the active components were eluted with 50% aqueous methanol containing 0.1% hydrochloric acid. The active eluates were neutralized by passage through a Dowex-44 (OH^- type) column and they were concentrated under reduced pressure at 30°C. The concentrated solution was lyophilized, the crude powder was dissolved in methanol, the insoluble residue was separated by filtration, and the clear methanol solution was concentrated under reduced pressure. The S-15-1 antibiotic was precipitated as the hydrochloride by addition of acetone. The precipitate was separated, washed with acetone and dried.

Futher purification was done by a cellulose column with *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) solvent system. Active fractions were concentrated, and the antibiotic acetate was converted to the sulfate salt utilizing an Amberlite IRC-50 (H^+ type) column. The solution was concentrated under reduced pressure at 30°C and lyophilized. Two hundred milligrams of S-15-1 sulfate were thus obtained as a white powder. The purified S-15-1 sulfate was detected in thin-layer chromatograms as a clear single spot by ninhydrin tests and by antiviral and antimicrobial tests. Cellulose thin-layer chromatograms were developed with *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) and *n*-butanol - acetic acid - water (2 : 1 : 1). Silica gel plates were developed with chloroform - methanol - 17% aqueous ammonia (2 : 1 : 1, upper layer) and pyridine - acetic acid - water (50 : 35 : 15).

Physico-chemical and Biological Properties of S-15-1

S-15-1 sulfate is soluble in water, insoluble in methanol, ethanol, acetone, ethylacetate, chloroform, ethylether, benzene, and hexane. It is stable at powder state and at 90°C for 10 minutes with the solution of pH 2.0, but little activity remains when it is treated at pH 8.0 and 90°C for 10 minutes.

It melts at 161~171°C under decomposition. It shows only end absorption in the ultraviolet spectrum. The infrared absorption spectrum in KBr disc is presented in Fig.1. It has characteristic absorption bands at the following frequencies: 3400, 1718, 1655, 1615, 1559, 1495, 1395, 1335, 1315, 1250, 1220, 1193, 1080, 1055, 1005, 890, and 780 cm^{-1}.

Fig. 1. Infrared spectrum of S15-1 sulfate in KBr-disc.

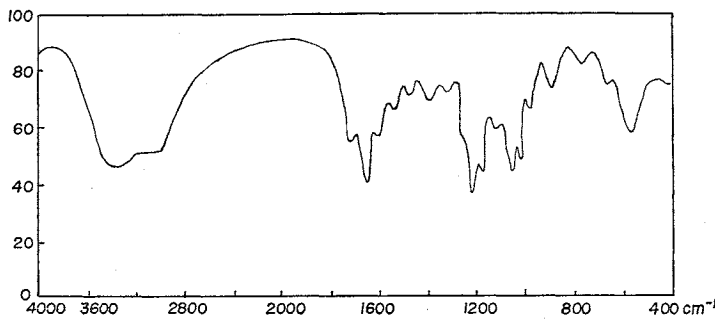

Table 3. Thin-layer chromatography of S-15-1 sulfate

A) Cellulose plate

Solvent systems	Rf values
n-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)	0.08
n-Butanol - acetic acid - water (2 : 1 : 1)	0.15
Wet *n*-butanol containing 2 % (w/v) *p*-toluene-sulfonic acid	0.15

B) Silica gel G plate

Solvent systems	Rf values
Chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1) (upper layer)	0.28
n-Butanol - acetic acid - water (2 : 1 : 1)	0.05

Table 4. Antiviral activity of S15-1 sulfate by the agar diffusion method

Concentration (mcg/ml)	PIZ
2,000	27
100	19
80	15
30	10
10	—

Diameter of plaque-free inhibition zones (PIZ) were measured and expressed in mm.

The optical rotation of the S-15-1 hydrochloride is $[\alpha]_D$ −26° (*c* 1.0, water). Elemental analysis is as follows:

Calcd. for $C_{22}H_{49}N_9O_{11}\cdot 2HCl$:

C 38.37, H 7.41, N 18.31, O 25.58, Cl 10.32.

Found:

C 38.30, H 7.30, N 18.80, O 24.50, Cl 10.40.

The antibiotic is positive to MOLISCH, SAKAGUCHI (strong), ELSON-MORGAN (pink), FEHLING, anthrone, maltol (weak) and ninhydrin tests, but negative to biuret and orcinol tests. The results of thin-layer chromatography are shown in Table 3.

The antiviral activity of the antibiotic is shown in Table 4. No cytotoxicity was observed at the higher concentration. The antimicrobial activities were examined using the agar streak dilution method and the results are shown in Table 5. It shows inhibitory activity against gram-positive and gram-negative bacteria, and some yeast strains. The intravenous LD_{50} in mice (ddY) is 150 mg/kg. However, as occurs with streptothricin, delayed toxicity begins to appear after 2 days at 100 mg/kg, and after 3 days at 50 mg/kg.

Table 5. Antimicrobial activity of S15-1 sulfate

Test organisms*	M.I.C. (mcg/ml)
Staphylococcus aureus FDA 209P	0.78
Staphylococcus aureus FDA 209P (ST-R, SM-R)	>50.00
Sarcina lutea PCI 1001	6.25
Bacillus subtilis PCI 219	0.78
Bacillus cereus IFO 3001	25.00
Proteus vulgaris IFO 3851	1.56
Escherichia coli K-12	3.13
Escherichia coli B (SM-R)	3.13
Escherichia coli (KM-R)	3.13
Escherichia coli W3630 (SA, PC, TC, CP, SM-R)	1.56
Escherichia coli (ST-R)	>50.00
Pseudomonas aeruginosa IFO 3448	>50.00
Mycobacterium 607 (SM-R)	6.25
Saccharomyces cerevisiae HANSEN Kyokai 6	>50.00
Candida albicans IAM 4888	50.00
Cryptococcus neoformans IAM 4514	25.00
Penicillium chrysogenum THOM FAT-917	>50.00
Aspergillus niger VAN THIEGHEN	50.00

* -R: resistant.
ST: streptothricin, SM: streptomycin, KM: kanamycin, SA: sulfonamide, PC: penicillin G, TC: tetracycline, CP: chloramphenicol.

Discussion

All *Streptomyces* isolated were tested for cytotoxic, antiviral, and antimicrobial activity. Many strains produced cytotoxic antibiotics, but few strains produced only antiviral antibiotics. One isolate, *Streptomyces* S-15-1, produced a noncytotoxic, antiviral antibiotic with broad antimicrobial activity. This water-soluble basic antibiotic was differentiated from the known antibiotics by its physico-chemical and biological properties. The antibiotic

gave positive SAKAGUCHI (strong), maltol (weak), nynhydrin, MOLISCH, and anthrone reactions, and negative orcinol and biuret reactions. It was differentiated from streptomycin group antibiotics, because of a negative ninhydrin reaction and its antiviral activity and the activity against *Candida albicans*.

The positive SAKAGUCHI and maltol reactions, and negative orcinol reaction differentiate the antibiotic from kanamycins, neomycins, paromomycins, zygomycins, hydroxymycins, and catenulin.

It was also differentiated from streptothricin group antibiotics (Tables 6 and 7). New streptothricin-like antibiotics which were recently found and reported by various groups have higher Rf values than that of kanamycin on silica gel thin-layer chromatogram developed with the upper layer from a mixture of chloroform - methanol - 17 % aqueous ammonia (2 : 1 : 1). Antibiotic S-15-1 has a much smaller Rf value than that of kanamycin. Streptothricins and racemomycins gave negative SAKAGUCHI and maltol reactions.

Antiviral antibiotics such as amidinomycin, phagolessin, phagocidin, grasseriomycin, and myxoviromycin were differentiated by color reaction and antimicrobial activity. These antiviral antibiotics are maltol negative. Grasseriomycin is inactive against *Candida albicans*. Amidinomycin, phagolessin, phagocidin, myxoviromycin, and amidinomycin are weakly active against gram-positive and gram-negative bacteria.

Table 6. Chromatographic comparison of S15-1 with other known antibiotics

Antibiotics	Rf values						
	A				B		C
	TLC			PPC	TLC	PPC	TLC
	I	II	III		I		III
S15-1	0.05	0.08	0.50	0.10	0.15	0.00	0.28
Boseimycin[3]			0.75			0.08	
BD-12[4]				0.54		0.29	0.78
BY-81[4]		0.36[12]		0.45		0.17	0.52[12]
Citromycin[12]		0.35					0.52
LL-AC541[12]		0.35		0.50			0.52
Racemomycin		0.25		0.38	0.20		0.36
Sclerothricin[8]							0.78
SF-701[11]	0.66	0.33[12]			0.23		0.72
Streptothricin	0.26		0.72	0.38			0.26
Viomycin	0.21			0.28			0.11[13]
Yazumycin[11]			0.64			0.09	0.3～0.33

A : *n*-Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)
B : Wet *n*-butanol containing 2.0 % (w/v) *p*-toluenesulfonic acid
C : Chloroform-methanol - 17 % aqueous ammonia (2 : 1 : 1) (upper layer)
I : Cellulose MN 300 powder
II : Avicel SF plate
III : Silica gel G

Table 7. Comparison of S15-1 with other known antibiotics

Antibiotics	Color reactions					
	Biuret	FEHLING	Maltol	MOLISCH	Ninhydrin	SAKAGUCHI
S15-1	−	+	+	+	+	+
Boseimycin[3]	+	−	−	+	+	+
BD-12[4]	?	?	−	−	+	−
BY-81[4]	−	?	−	−	+	−
Citromycin[5]	−	−	−		+ (in pyridine)	−
EC-749C[6]		−			−	−
LL-AC 541[7]	+	+	−	−	−	−
Racemomycin	−	+	−	−	+	−
Sclerothricin[8]	−	−	−	−	+	±
SF-701[9]	−	+		−	+	−
Streptothricin	+	+	−	−	+	−
Viomycin[10]	+	+	−	−	+	+
Yazumycin[11]	+ (weak)	+	−		+	+

+ : Positive − : Negative ± : Slightly colored ? : Doubtful

Accordingly it can be concluded that the antibiotic S-15-1 is a new antiviral and antimicrobial antibiotic.

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