REDUCTIOMYCIN, A NEW ANTIBIOTIC
I. TAXONOMY, FERMENTATION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES

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A new antibiotic, reductiomycin was isolated from the culture broth of *Streptomyces griseorubiginosus*. The antibiotic has the molecular formula $C_{14}H_{15}O_6N$ (M.W. 293). Reductiomycin is active against Gram-positive bacteria, fungi, and Newcastle disease virus.

In the course of our screening for new antiviral antibiotics employing an agar diffusion method,[1] an actinomycete strain S551 identified as *Streptomyces griseorubiginosus* was found to produce a new antibiotic. The antibiotic named reductiomycin was active against Newcastle disease virus (NDV), Gram-positive bacteria and fungi. In this paper, the taxonomy of the producing strain, the fermentation, the isolation, the characterization and biological activities of reductiomycin are described.

The structural elucidation by spectroscopic studies will be the subject of the following paper.

Taxonomy

An actinomycete strain S551 was isolated from a soil sample collected in Osaka-shi, Japan. Since the reductiomycin-producing strain S551 was found to represent a species of *Streptomyces*, the taxonomic characterization was carried out according to the method used in the International Streptomyces Project (ISP).[2]

Microscopic observation showed abundant aerial mycelium with flexious spore chains. The mature spore chains were generally long with 10~30 or more spores per chain. The spores were oval in shape (0.9~1.3 $\mu \times 0.5 \sim 0.6$ μ) and possessed a smooth surface as seen by electron microscope.

The aerial mycelium was gray colored on agar media, thus a member of the color-series "gray". The golden color occasionally occurred in aged cultures and the reverse mycelium color was golden to brown. Melanin was produced on some agar media including peptone - yeast extract - iron agar and tyrosine agar. These cultural characteristics are summarized in Table 1.

The following carbohydrates supported growth as the sole carbon source: D-glucose, D-fructose, D-mannitol and raffinose. No or little growth was observed on D-arabinose, D-xylose, *m*-inositol and cellulose. Gelatin was not liquefied and milk was peptonized by the strain. The strain showed good growth and sporulation at temperatures between 25~35°C.

Analysis of above results indicates that the strain S551 can be identified as *Streptomyces griseorubiginosus* according to the method of KUSTER's classification.[3] Although there are some discrepancies in physiological characteristics such as carbohydrate utilization described in literatures,[4,5] we considered this to be minor and designated the strain S551 as *Streptomyces griseorubiginosus* S551.

The strain S551 has been deposited at Northern Regional Research Laboratories, Peoria, Illinois, U. S. A. and has been assigned the accession number NRRL 11268.

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Table 1. Cultural characteristics of the strain S551.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
CZAPECK's agar (Waksman No. 1)	poor, flat	colorless (a)	colorless (a)	none
Glucose-asparagine agar (Waksman No. 2)	moderate, raised	light ivory (2ca)	light yellow (1 1/2ea)	none
Yeast-malt extract agar (ISP No. 2)	good, raised	cream (1 1/2ca)	mustard gold (2ne)	amber (3pc)
Oatmeal agar (ISP No. 3)	good, raised	putty (1 1/2ec)	golden brown (3pg)	mustard gold (2ne)
Inorganic salts-starch agar (ISP No. 4)	good, raised	cream (1 1/2ca)	light yellow (1 1/2ea)	butter yellow (1 1/2ga)
Glycerol-asparagine agar (ISP No. 5)	moderate, flat	light ivory (2ca)	light yellow (1 1/2ea)	none
Peptone-yeast extract agar (ISP No. 6)	poor, raised	none	dull gold (2ng)	clove brown (3ni)
Tyrosine agar (ISP No. 7)	good, raised	cream (1 1/2ca)	mustard brown (2ni)	antique gold (1 1/2ne)
Nutrient agar	moderate, flat	none	bright gold (2nc)	amber (3pc)

Color designation are according to Color Harmony Manual.[6]

Fermentation

A loopful of the spore suspension obtained on BENNETT's agar medium was inoculated into 100 ml of a seed medium consisting of 2% soluble starch, 2% glutenmeal, 2% Pharmamedia, 2% Ebios, 0.07% $MgSO_4 \cdot 7H_2O$ and 0.3% $CaCO_3$ (pH 6.5) in a 500-ml Sakaguchi flask. After incubated at 30°C for 45 hours on a reciprocal shaker, 300 ml of the seed cultures were transferred into 15 liters of the same medium in a 30-liter jar fermentor. Fermentation was carried out at 30°C for 72 hours under aeration (14 liters/minute) and with agitation (300 r.p.m.).

Actinomycin (unidentified) was coproduced along with reductiomycin by the strain S551. The separation of reductiomycin and actinomycin was accomplished by thin-layer chromatography on silica gel plates (E. Merck, Art. 5715). With ethyl acetate Rf values of reductiomycin and actinomycin were 0.52 and 0.22, respectively. Reductiomycin was visualized by exposing the plates to UV light or iodine vapour.

The time course of the fermentation is shown in Fig. 1. Total activities reached a maximum at 48 hours after inoculation. Reductiomycin was detected after 24 hours and reached the maximum concentration at 72 hours.

Isolation and Purification

A flow diagram for the isolation of reductiomycin is given in Fig. 2. Reductiomycin readily crystallized in acetone or ethyl acetate.

Physico-chemical Properties

Pure reductiomycin was obtained as yellow needles, which melted at 215°C with decomposition, $[\alpha]_D^{23}+281°$ (c 0.30, acetone). The high resolution mass spectrum revealed the molecular ion at m/z 293.280 ($C_{14}H_{15}O_6N$). The elemental analysis was as follows: C 57.34%, H 5.12%, N 4.77%. Calcd.

Fig. 1. Time course of fermentation.

Fermentation was carried out in a 30-liter jar fermentor using the medium indicated in the text at 30°C with agitation of 300 rpm and aeration of 14 liters/minute. Total activity was determined by disc method on nutrient agar, using *Bacillus subtilis* IAM 1026 as the test organism and was expressed by diameters of antibacterial zones.

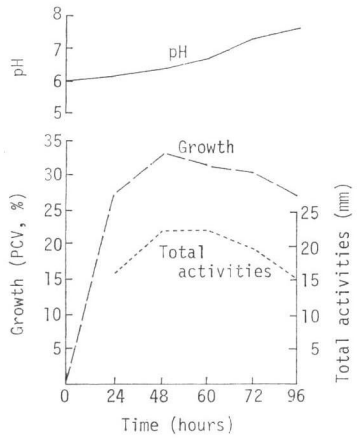
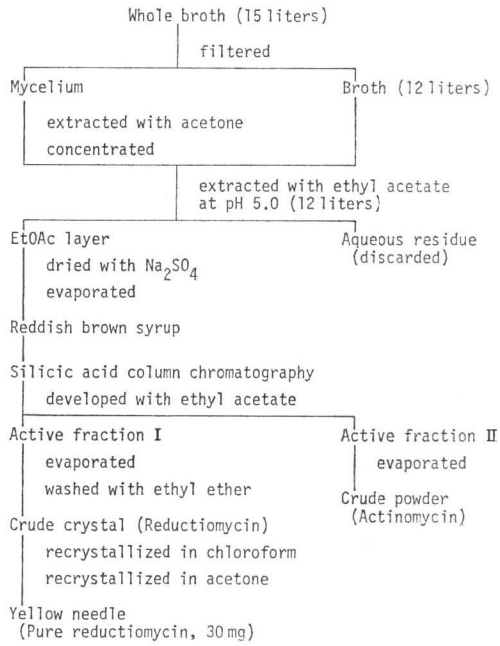


Fig. 2. Isolation procedure for reductiomycin



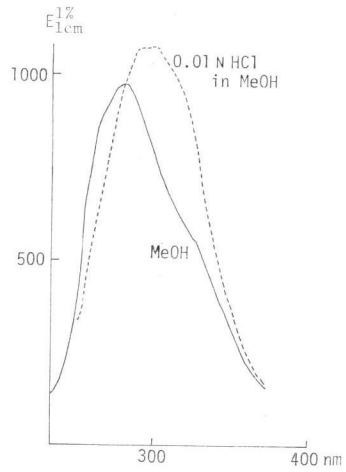
for $C_{14}H_{15}O_6N$: C 57.36%, H 5.25%, N 4.73%.

Reductiomycin is readily soluble in dimethylsulfoxide, soluble in chloroform, ethyl acetate and acetone, slightly soluble in methanol, and practically insoluble in ethy ether, petroleum ether and water.

Reductiomycin is unstable in alkaline solution (> pH 8), but it is stable in neutral or acidic solution even when heated at 60°C for 30 minutes (pH 2~7).

It gave positive color reactions with ferric chloride, 2,4-dinitrophenyl hydrazine, nitroprusside and Ehrlich reagents, but negative with ninhydrin, pine-shaving, SAKAGUCHI and SCHIFF's reagents.

Fig. 3. UV spectrum of reductiomycin.



The UV spectrum shows one broad peak with a maximum at 282 nm (ε 28660) and two shoulders at 265 nm (ε 25870) and 326 nm (ε 15470) in methanol as shown in Fig. 3.

The infrared absorption spectrum in nujol (Fig. 4) exhibits characteristic bands for acetyl C=O (1730 cm^{-1}) and enol form of 1,3-diketone (1600 cm^{-1}).

Fig. 4. IR spectrum of reductiomycin (in nujol).

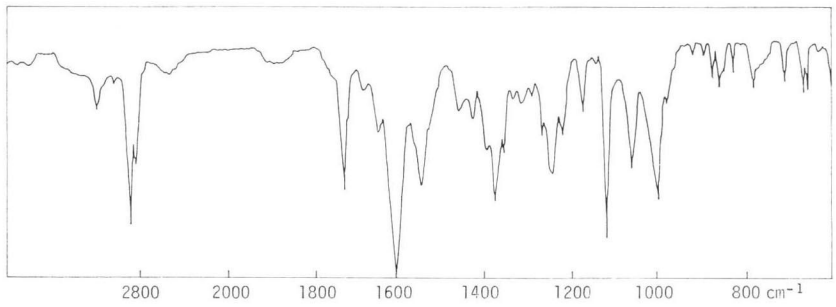


Table 2. Anti-NDV activity of reductiomycin (agar-diffusion method).

Concentration (mcg/ml)	Anti-NDV activity (mm)
1,000	27
500	23
250	18
125	18
63	16
31	14
16	±
8	−

Monolayer cultures of chick embryo fibroblasts in Petri dishes were infected with NDV.

Antiviral activity was determined by the method described in reference 2, and was expressed by diameters of antiviral zones.

Table 3. Anti-NDV activity of reductiomycin (tube method).

Concentration (mcg/ml)	Cytotoxicity	Anti-NDV activity (inhibition %)
100	##	>95
50	##	>95
25	#	>95
13	+	>95
6	+	77
3	−	50
1.5	−	25
0.75	−	0

Cell sheets in test tubes were infected with NDV at an input multiplicity of 10 plaque forming unit/cell. Virus production was determined by HAU titration, and antiviral activity was expressed as % inhibition. Cytotoxicity was observed microscopically at the same time and expressed as follows:

−; without cytotoxicity, +; slight cytotoxicity, #; moderate cytotoxicity; ##; severe cytotoxicity.

Biological Activities

Antiviral activity was determined by plaque-inhibition using an agar-diffusion method. Reductiomycin inhibited multiplication of NDV in cultured chick embryo fibroblasts (Table 2). Reductiomycin was active against NDV at 31 μg/ml, but decreased incorporation of neutral red into cells was observed. As shown in Table 3,

Table 4. Antimicrobial spectra of reductiomycin (paper disc method).

Test organisms	MIC (μg/ml)
Bacillus subtilis IAM 1026	31
Sarcina lutea IAM 1099	125
Staphylococcus aureus FAD 209P	63
Escherichia coli ATCC 3655	500
Serratia marcescens IAM 1022	1,000
Proteus vulgaris HX 19 IAM 1025	1,000
Pseudomonas aeruginosa IAM 1156	1,000
Aspergillus niger IAM 2026	125
Aspergillus oryzae NRRL 692	63
Penicillium chrysogenum Q 176	63
Mucor spinescens IAM 6071	500
Fusarium moniliforme IAM 5062	125
Myrothecium verrucaria IAM 5063	63
Trichophyton mentagrophytes IAM 5064	1
Trichoderma T-1 ATCC 9645	8
Chaetomium globosum ATCC 6025	250
Microsporum gypseum IFO 5948	125
Alternaria solani IFO 5924	4
Cladosporium herbarum IAM 5059	31
Candida albicans IAM 4888	1,000
Saccharomyces cerevisiae IAM 4485	1,000
Saccharomyces rouxii M-9	1,000

when tested by the tube method, virus multiplication was inhibited completely at 13 μg/ml. Slight cytotoxicity was detected at 6 μg/ml and partial or complete destruction of cell sheets was observed at 50 μg/ml.

The antimicrobial activities of reductiomycin are shown in Table 4. This test was carried out by paper disc method using glucose-nutrient agar. Reductiomycin inhibited Gram-positive bacteria, fungi and certain yeasts. Against *Trichophyton mentagrophytes* and *Alternaria solani*, it was active at comparatively low concentration.

The acute toxicity (LD_{50}) of reductiomycin by the intraperitoneal route in mice was about 80 mg/kg.

Acknowledgement

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