Resormycin, a Novel Herbicidal and Antifungal Antibiotic Produced by a Strain of *Streptomyces platensis*

I. Taxonomy, Production, Isolation and Biological Properties

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Resormycin, a novel herbicidal and antifungal antibiotic, was isolated from the cultured broth of streptomycete strain. The strain was isolated from a soil collected at Yokohama-shi, Kanagawa Prefecture, Japan, and identified as *Streptomyces platensis* MJ953-SF5.

Resormycin was purified by active charcol, Amberlite IRC-50, Amberlite CG-50 and Sephadex LH-20 column chromatographies. Resormycin markedly inhibited the growth of monocotyledonous and dicotyledonous weed. The antibiotic showed antimicrobial activity against phytopathogenic fungi.

In the course of screening for new herbicidal and new anti-microbial substances from microorganisms, we found that a streptomycete strain which was isolated from a soil collected at Yokohama, Kanagawa, Japan, produced new tripeptide antibiotic, named resormycin (Fig. 1), consisting of three rare amino acids. Resormycin showed marked inhibition of the growth of weeds. The compound showed antifungal activity against phytopathogenic fungi but not against yeast and bacteria.

In this paper, we describe the identification of the producing organism together with the isolation, fermentation and biological activities of resormycin. Physico-chemical properties and structure elucidation of the compound will be described in an accompanying paper[1].

Materials and Methods

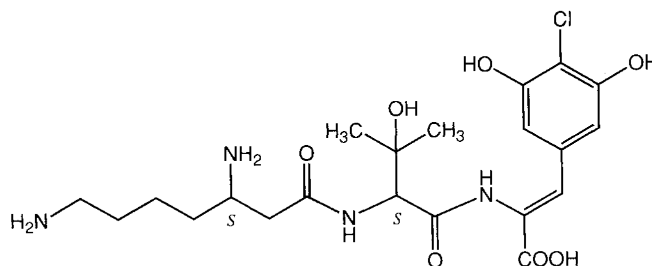
Taxonomy

Resormycin producing organism, strain MJ953-SF5, was isolated from a soil sample collected at Yokohama, Kanagawa, Japan. Morphological, cultural and physiological properties of the strain MJ953-SF5 were examined according to the methods described by SHIRLING and GOTTLIEB[2], and WAKSMAN[3]. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Model S-570, Hitachi) after strain MJ953-SF5 was incubated on sucrose - nitrate agar and inorganic salts - starch agar (ISP No. 4) at 27°C for 10 days. Chemical analyses of cell wall was analyzed using thin layer chromatography (TLC) according to the method of STANECK and ROBERTS[4]. Menaquinone was performed with the methods of TAMAOKA *et al.*[5]

Fermentation

A slant culture of the resormycin-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2%, dextrin 2%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki Co.) 0.5%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 2 days. Two ml of the seed culture was transferred into a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium which consisted of glucose 1.0%, glycerol 1.0%, sucrose 1.0%, oatmeal 0.5%, soybean meal 2.0%, press yeast 1.0%, casamino acids 0.5%, and $CaCO_3$ 0.1%

Fig. 1. Structure of resormycin (1).



in deionized water (pH 7.4 before sterilization). The fermentation was carried out at 27°C for 4 days on a rotary shaker.

Analytical Procedure

Resormycin content in fermentation broth and purification steps was monitored by reversed phase HPLC and silica gel TLC. HPLC was performed as follow: CAPCELL PACK C_{18} column (4.6 × 150 mm, Shiseido Co., Ltd., Japan; mobile phase, 40% aqueous acetonitrile - 1% formic acid; flow rate, 1.5 ml/minute; column temperature, 40°C; detection, UV (300 nm). It was eluted at 5.7 minutes. TLC was performed with Kieselgel 60 F_{254} (Art. No. 5715, Merck) developed with $CHCl_3$ - MeOH - conc NH_4OH - H_2O (1:4:2:1). Spots on TLC were detected by ninhydrin reaction. Rf value of resormycin was 0.65.

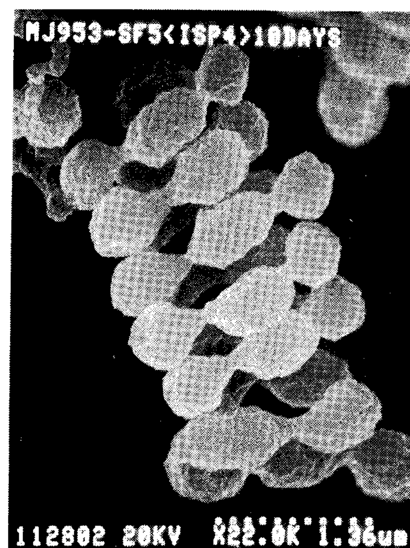
Biological Activity

The germination and growth inhibition assay was performed using lettuce seeds (*Lactuca sativa*). Absorbent cotton in each well of a 24-well flat-bottomed tissue culture plate (Falcon 3047, Becton Dickinson Co.) was fully moistened with a sample solution (1 ml) which was diluted with distilled water. The seeds were placed on the absorbent cotton and incubated in a growth cabinet at 25°C for 16 hours under illumination of 2000 lux and 8 hours in darkness. After 5-days incubation, the herbicidal activity was observed.

The plants used for herbicidal test were as follows; *Digitaria adscendens* (Henry crabgrass), *Setaria viridis* (Green foxtail), *Alopecurus aequalis*, *Poa annua* (Annual bluegrass), *Amaranthus viridis* (Livid amaranth), *Polygonum lapathifolium* (Pale smartweed), *Bidens pilosa* (Hairy beggarticks), *Portulaca oleracea* (Common purslane), *Chenopodium album* (Common lambsquarters), *Senecio vulgaris* (Common groundsel), *Brassica arvensis* (Wild mustard), *Gossypium hirsutum* (Cotton), *Glycine max* (Soy bean), and *Zea mays* (Corn). The plants, 18-day old seedlings, were treated with resormycin through foliage application. The herbicidal effects were observed 21 day after the treatment.

The minimum inhibitory concentrations (MIC) of resormycin was examined by serial agar dilution method using potato sucrose agar and Nutrient - 1% glucose agar for yeasts and fungi and Mueller-Hinton agar (Difco) for bacteria. The MIC was observed after 96 hours incubation at 27°C for antiphytopathogenic fungi and 42 hours incubation at 27°C for yeast and fungi and 18 or 42 hours incubation at 37°C for bacteria.

Photo. 1. Scanning electoron micrograph of spore chains of *Streptomyces platensis* MJ953-SF5 grown on ISP-4 agar for 10 days at 27°C.



Results

Taxonomic Features of Strain MJ953-SF5

Strain MJ953-SF5 produced well-branched vegetative mycelia. This strain formed long aerial hyphae which bore spirals of 3 to 6 turns. Mature spore chain consisted of 10 to 50, or more spores. The spore was oval with smooth surface and $0.5 \times 0.6 \sim 0.8 \times 1.0\,\mu m$ in size (Photo. 1). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain MJ953-SF5 on various agar media are shown in Table 1. The aerial mycelia were pinkish gray to light gray. The vegetative mycelia were pale yellow to pale yellowish brown or grayish red. The soluble pigments were a shade of pale red to faint, brownish. Physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature ranges for growth of the strain were 20°C to 37°C. The optimal temperatures for growth of strain MJ953-SF5 were between 24°C and 27°C.

Whole-cell hydrolysates of strain MJ953-SF5 contained LL-diaminopimelic acid. Major components of menaquinones were MK-9 (H_6) and MK-9 (H_8).

These taxonomic properties suggested that strain MJ953-SF5 belonged to the genus *Streptomyces*. We searched the taxonomic data of known *Streptomyces* species in the ISP descriptions by SHIRLING and GOTTLIEB[6~9], the International Journal of Systematic Bacteriology and the type cultures. In the result, strain

Table 1. Cultural characteristics of strain MJ953-SF5.

Medium	Growth	Aerial mycelium	Soluble pigmemt
Sucrose-nitrate agar	Colorless	Thin, gray [2ih, Dk Covert Gray] ~light brownish gray [3ig, Beige Brown]	None
Yeast extract-malt extract agar (ISP No. 2)	Pale orange [3gc, Lt Tan]~pale red [6le, Cedar]	Pinkish white [7ba, Pink Tint] ~light gray [d]~Dark brownish gray [2nl, Covert Brown][a]	Shade of pale red ~brownish
Oatmeal agar (ISP No. 3)	Colorless~pale orange [4ec, Bisque]	Light gray [d]~gray [i, Gray] ~dark brownish gray [3nl, Dk Brown]	Faint, brownish
Inorganic salts-starch agar (ISP No. 4)	Pale yellowish brown [2le, Mustard~3le, Cinnamon]	White~pinkish gray [5ca, Flesh Pink]~gray [f][a]	Shade of pale pink
Glycerol-asparagine agar (ISP No. 5)	Pale yellow [2ca, Lt Ivory]~pale pink [6 1/2gc, Dusty Coral]	Pinkish gray [6ec, Powder Rose] ~light gray [2fe, Covert Gray][a]	Faint, brownish
Tyrosine agar (ISP No. 7)	Pale yellow [2ea, Lt Wheat] ~pale purplish pink [7ec, Rose Mist]~grayish red purple [8ge, Dusty Mauve]	Pinkish white [7ba, Pink Tint] ~light gray [d][a]	Brownish
Glucose-asparagine agar	Pale yellow [2gc, Bamboo] ~pale yellowish brown [2ne, Mustard Gold]	White~grayish white [b, Oyster White]	Faint, brownish
Nutrient agar	Pale yellow [2gc, Bamboo]	Thin, white	None
Starch agar	Grayish red [6 1/2lg, Lt Rose Brown~6 1/2ni, Rose Brown]	Pinkish gray [7ec, Rose Mist]	Pale reddish brown

Observation after incubation at 27 °C for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.[10)]
[a] Hygroscopic areas are found in the aerial mycelium.

MJ953-SF5 was closely related to *Streptomyces platensis*[6)] except for small differences in milk peptonization (Table 3).

Therefore, the strain was identified as *Streptomyces platensis* and designated *Streptomyces platensis* MJ953-SF5. Strain MJ953-SF5 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba, Japan, under the accession No. FERM P-15335.

Table 2. Physiological characteristics of strain MJ953-SF5.

Temperature range for growth (°C)	20~37
Optimum temperature (°C)	24~27
Formation of melanoid pigment	
ISP No. 1	Negative
ISP No. 6	Negative
ISP No. 7	Negative
Liquefaction of	
gelatin	Weakly positive
glucose peptone gelatin	Negative
Coagulation of milk	Positive
Peptonization of milk	Weakly positive
Hydrolysis of starch	Positive
Reduction of nitrate	Negative
Utilization of	
L-Arabinose	(−)
D-Xylose	(−)
D-Glucose	+
D-Fructose	±
Sucrose	±
Inositol	+
Rhamnose	−
Raffinose	(+)
D-Mannitol	+

+: Utilization, (+): probably utilization, ±: doubtful, (−): probably no utilization, −: no utilization.

Fermentation and Isolation

The active substance was monitored by the antifungal activity against *Pyricularia orizae* P-2 and HPLC analysis during the purification process. A typical time course of resormycin production in a 500-ml baffled Erlenmeyer flask fermentation is shown in Fig. 2. The production of resormycin started after 2 days of cultivation and reached a maximum (*ca.* 100 mg/liter) after 4-day's incubation.

The culture broth (10 liters) was filtered by using Hyflo Super-Cel (John-Manville Co., U.S.A.) in filter aid. The culture filtrate was adjusted to pH 7.0 with 6 M HCl and applied to an active carbon column. The column was washed with deionized water (1500 ml) and 0.1 M HCl

Table 3. Comparison of taxonomic characteristics of strain MJ953-SF5 with *S. platensis*.

	MJ953-SF5	*S. platensis* ISP 5041
Aerial mycelium morphology	Spiral	Spiral
Spore surface	Smooth	Smooth
Color of aerial mycelium	Grayish white ~ pinkish white ~ light grayish white	White ~ pinkish white ~ light grayish white
Color of growth	Pale yellow ~ pale yellowish brown ~ dull red	Pale yellow ~ pale yellowish brown ~ dull red
Soluble pigment	Pale pink ~ pale brown	Pale pink ~ pale brown
Formation of melanoid pigment		
ISP No. 1	Negative	Negative
ISP No. 6	Negative	Negative
ISP No. 7	Negative	Negative
Liquefaction of		
gelatin	Weakly positive	Weakly positive
glucose peptone gelatin	Negative	Negative
Coagulation of milk	Positive	Positive
Peptonization of milk	Weakly positive	Negative
Hydrolysis of starch	Positive	Positive
Reduction of nitrate	Negative	Negative
Utilization of		
L-Arabinose	(−)	(−)
D-Xylose	(−)	(−)
D-Glucose	+	+
D-Fructose	±	+
Sucrose	±	+
Inositol	+	+
Rhamnose	−	−
Raffinose	(+)	+
D-Mannitol	+	+

+: Utilization, (+): probably utilization, ±: doubtful, (−): probably no utilization, −: no utilization.

(1500 ml). The active principle was eluted with 50% aqueous acetone. The active eluate was concentrated under reduced pressure and neutralized with 1 N NaOH. The concentrate was charged on a column of Amberlite IRC-50 ($H^+ : NH_3^+ = 1:1$). After the column was washed with distilled water, the active principle was eluted with 1 M aqueous NH_4OH. The active fractions were collected and concentrated *in vacuo* to give a brownish oil. The crude oil was further chromatographed on an Amberlite CG-50 column developing with distilled water. The active fractions were collected and concentrated to give crude resormycin as a brownish powder. The crude resormycin was dissolved with 1 M HCl and adjusted to pH 3. The solution was further purified by Sephadex LH-20 chromatography developing with 50% aqueous MeOH. Active fractions were collected and concentrated *in vacuo* yielding a white powder of pure resormycin (0.20 g) as HCl salt (Fig. 3).

The structure of resormycin is shown in Fig. 1. The studies on the structure determination of this antibiotic will be reported in an accompanying paper[1].

Fig. 2. A typical time cource of resormycin production.

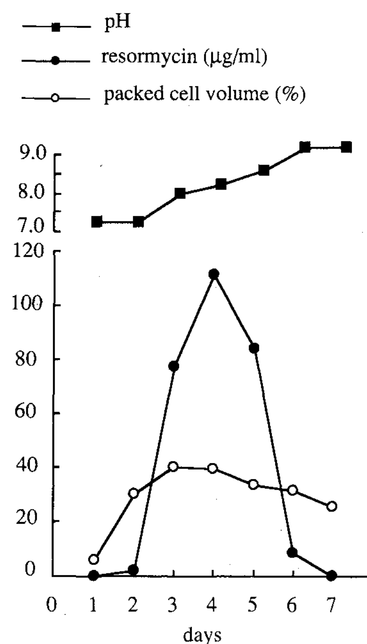
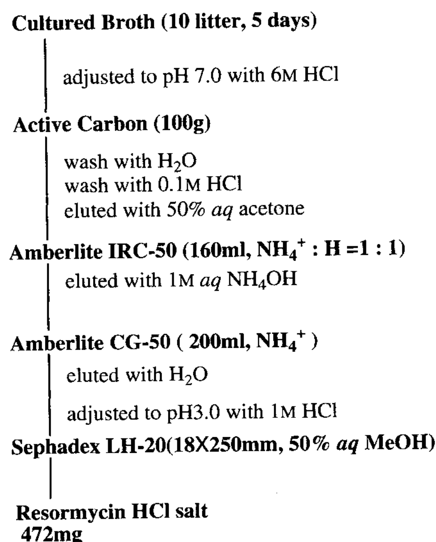


Fig. 3. Isolation and purification procedure of resormycin.



Biological Activity

The growth of lettuce seedling treated with resormycin at 6.25 ppm was completely inhibited. The herbicidal activity of resormycin against monocotyledonous and dicotyledonous weeds by foliar application is shown in Table 4. Resormycin showed strong herbicidal activity against various plants, especially dicotyledonous weeds. However, resormycin did not show herbicidal activity against all weeds used in this study, by soil application.

Resormycin exhibited antifungal activity against some phytopathogenic fungi but was inactive against bacteria and yeast. The antimicrobial activity of resormycin is shown in Table 5.

Table 4. Herbicidal activity of resormycin by foliar application.

Plants	ppm		
	1000	500	250
Digitaria adscendens	5	4	2
Setaria viridis	5	3	2
Alopecurus aequalis	NT	2	1
Poa annua	NT	2	2
Amaranthus viridis	5	5	4
Polygonum lapathifolium	5	3	2
Bidens pilosa	5	5	5
Portulaca oleracea	NT	4	4
Chenopodium album	NT	4	3
Stellaria media	NT	3	2
Senecio vulgaris	NT	1	1
Brassica arvensis	NT	5	3
Zea mays	NT	0	0
Glycine max	NT	3	2
Gossypium hirsutum	NT	1	1

The extent of herbicidal activity was visually assessed as follow; 5: 100% killed, 4: 99~90% killed, 3: 89~70% killed, 2: 69~40% killed, 1: 39~20% killed, 0: 19~0% killed.
NT: not tested.

Table 5. Antimicrobial activities of resormycin.

Test organism	Med.	MIC (μg/ml)
Cercospora beticola	1	3.13
Diaporthe citri	1	<0.78
Pyricularia oryzae P-2	1	25
Colletotrichum lagenarium	1	6.25
Rhizoctonia solani	1	50
Botrytis cinerea B-22	1	6.25
Ustilago maydis	1	6.25
Staphylococcus aureus FDA209P	2	>100
Bacillus subtilis NRRL B-558	2	>100
Escherichia coli NIHJ	2	>100
Pseudomonas aeruginosa A3	2	>100
Mycobacterium smegmatis ATCC607	3	>100
Candida albicans 3147	4	>100
Saccharomyces cerevisiae F-7	4	>100
Criptococcus neoformans F-10	4	>100
Trichophyton asteroides 429	4	>100
Aspergillus fumigatus IFO9733	4	>100

Med. 1: Potato-sucrose agar 27°C 96 hours. Med. 2: Mueller Hinton agar 37°C 18 hours. Med. 3: Mueller Hinton agar 37°C 42 hours. Med. 4: Nutrient agar + 1% glucose 42 hours.

Discussion

Resormycin has three rare amino acids in the molecule. In particularly, 2-amino-3-(4-chloro-3,5-dihydroxy)phenylpropenoic acid moiety was rare in natural products. A few compounds having similar moiety were found in blood pigments of sea squirts and sponge such as tunichroms[11] and celenamides[12]. Several cinnamic acid derivatives have been known as plant growth regulator such as, 3,5-dihydroxy cinnamic acid[13], raphanusols[14,15], lespedezate[16] *etc.* from plant. However, they were not reported on herbicidal activity. Some amino acid-peptide type antibiotics with herbicidal activity from actinomycete are known such as homoalanosin[17], oxetin[18] and bialaphos[19,20]. The antibiotics were known as amino acid antimetabolites while resormycin did not show antimetabolite action on a minimal agar medium test. Mode of action of resormycin and structure-activity relationships are under investigation. The structure of resormycin is particularly interesting as a lead for the development of novel herbicides.

Acknowledgments

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