STUDIES ON ANTIBIOTIC O-2867, A NEW ANTIBIOTIC

TOMOYASU SATO*, KIYOZUMI YAMAGUCHI**, MICHIKO KATAGIRI*,
JUICHI AWAYA*, YUZURU IWAI*,
SATOSHI ŌMURA*,** and TOJU HATA*,**

The Kitasato Institute* and Kitasato University**,
Minato-ku, Tokyo, Japan

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A new antibiotic O-2867 inhibiting the growth of *Piricularia oryzae*, a plant pathogenic fungus of rice, was isolated from a *Streptomyces* sp. Antibiotic O-2867 is water-soluble, amphoteric and consists of 2 components, O-2867-α and β. O-2867-α was obtained as an amorphous powder and O-2867-β as colorless needles. Both antibiotics are active against *P. oryzae in vitro* but are not effective in pot tests using rice plants infected with *P. oryzae*.

In the course of our screening program for antibiotics active against plant pathogenic fungi, especially *Piricularia oryzae*, we found a new antifungal agent, antibiotic O-2867, in the culture fluid of a newly isolated *Streptomyces*, strain O-2867. Antibiotic O-2867 is a new amphoteric antibiotic and exhibits antifungal activity against *P. oryzae in vitro*. Its isolation and characterization are reported in this paper.

Materials and Methods

1. Media

(**1**) Rice straw extract medium: Rice straw, 100 g, was boiled in 1,000 ml of water and filtered through cloth. To this filtrate 0.2 % of yeast extract, 1 % of sucrose and 0.1 % of agar were added. As an assay medium for the antibiotic the agar content of the filtrate was increased from 0.1 % to 1.4 %.

(**2**) Potato extract medium: Potato extract medium for seed cultures was prepared as follows: potatoes 200 g were boiled in 1,000 ml of water and filtered through cloth. To the filtrate 1.0 % of glucose, 0.5 % of peptone and 0.3 % of agar were added. The medium was modified by adding 1.4 % of agar when it was used for bioassay.

2. Preparation of plates for bioassay

(**1**) Seed culture: *P. oryzae* cultured on a rice straw extract medium slant at 27°C for a few days was used to inoculate 125 ml of potato extract medium in a 500 ml flask; a 4-day culture incubated at 27°C on a reciprocating shaking machine was used as seed culture.

(**2**) Basal layer: Rice straw extract medium and potato extract medium were mixed in a 5 : 8 ratio respectively and streptomycin was added to a final concentration of 12.5 mcg/ml. Ten ml of this agar mixture was plated in a 90 mm Petri dish.

(**3**) Seed layer: One volume of seed culture was added to 5 volumes of basal layer medium and 4 ml of the mixture was plated on the basal layer.

(**4**) Assay method: Bioassay was carried out by the ordinary paper disc method. The inhibition zone was measured after incubation at 27°C overnight.

Characterization of Strain O-2867

Streptomyces strain O-2867 was isolated from a soil sample in 1969 at Ueda City, Nagano Prefecture, Japan. Its morphological characteristics were as follows : the aerial hyphae on oatmeal agar medium formed partly compact open spirals, but not whorls. Observation with the electronmicroscope showed oval spores with a smooth surface. Cultural characteristics of the strain on several diagonistic culture media were as follows : cream to yellowish brown growth with white to brownish white aerial mycelia ; no soluble pigment on organic media ; no starch hydrolysis and no gelatin liquefaction, but proteolytic activity on milk. Secretion of slime meterials was observed on certain media, such as glucose-asparagine agar and yeast extract-malt extract agar.

Antibiotic Production

Antibiotic O-2867 was produced in a medium composed of glucose 2%, peptone 0.5%, meat extract 0.5%, dry yeast 0.3%, NaCl 0.5% and $CaCO_3$ 0.3%, and adjusted to pH 7.0 before sterilization. The fermentation was carried out in a 30-liter stainless steel jar at 27°C under aeration of 10 liters air per minute and stirring at 250 r.p.m. Maximum potency of the antibiotic was obtained after 96 hours of cultivation. The fermentation pattern of strain O-2867 is shown in Fig. 1.

Isolation and Purification

The culture filtrate was adjusted to pH 2.0 with hydrochloric acid and treated with activated carbon. The

Fig. 1. Fermentation pattern for antibiotic O-2867

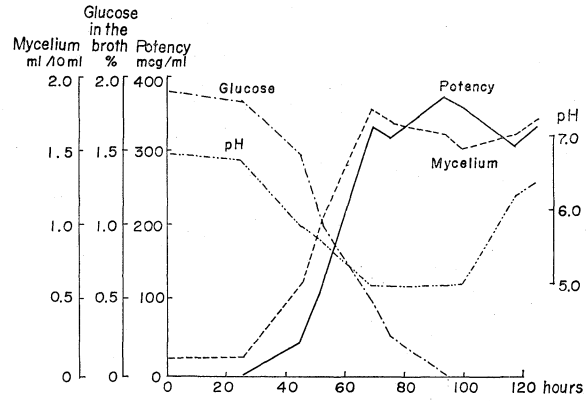
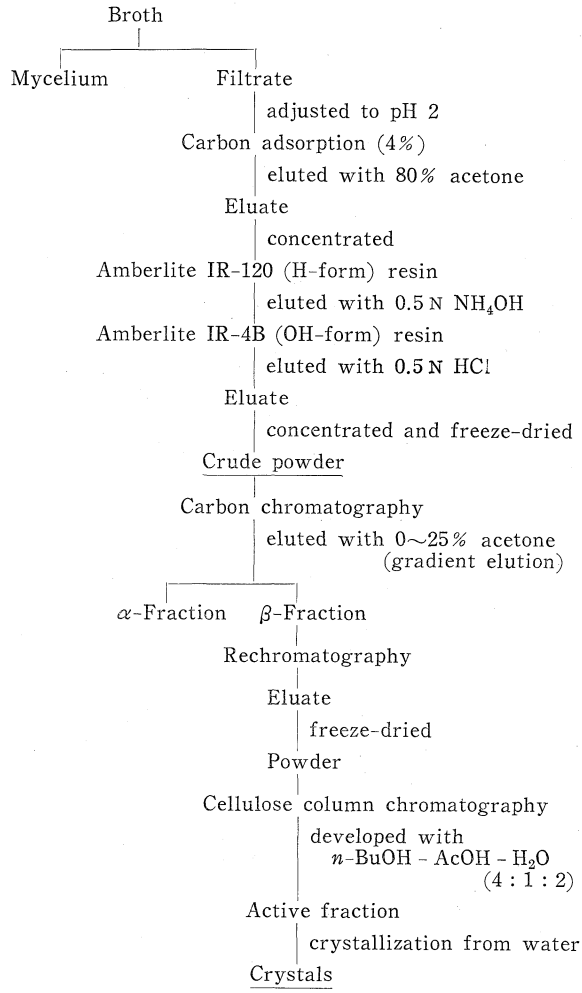


Fig. 2. Isolation and purification procedure



carbon cake was washed with water and the active substance was eluted with 80% aqueous acetone. The acetone solution was concentrated under reduced pressure and treated with cation exchange resin (IR-120, H-form). Active material was eluted with 0.5 N ammonium hydroxide and was adsorbed from the eluate on an anion exchange resin. A crude form of antibiotic O-2867 was recovered by eluting the resin with

Table 1. Physical and chemical properties of antibiotic O-2867-α and β

	α	β
Appearance	White powder	Colorless needle
Melting point	161~163.5°C	143~146°C
Nature	Amphoteric	Amphoteric
Solubility		
Soluble	Water, methanol	Water, methanol
Slightly soluble	Ethanol, acetone	Ethanol, acetone
Insoluble	Ethyl acetate, benzene, chloroform, hexane, ether	Ethyl acetate, benzene, chloroform, hexane, ether
Optical rotation	$[\alpha]_D^{24}$ −43° (*c* 1, water)	$[\alpha]_D^{24}$ −16° (*c* 0.8, water)
Color reaction		
Positive	Ninhydrin, EHRLICH	Ninhydrin, EHRLICH
Negative	Anthrone, ferric chloride, ELSON-MORGAN, DRAGENDORFF, tetrazolium salt	Anthrone, ferric chloride, ELSON-MORGAN, DRAGENDORFF, tetrazolium salt
Rf value*	0.66	0.53
Elementary analysis (%)	C : 43.59 H : 6.94 N : 11.72 O : 37.75	C : 43.37 H : 6.03 N : 13.71 O : 36.89
Molecular weight (RAST method)	380	313
Molecular formula	$C_{14}H_{25}N_3O_9$	$C_{10\sim11}H_{17\sim19}N_3O_7$
UV absorption	End absorption	End absorption

* Solvent system : *n*-butanol-acetic acid-water (4 : 1 : 2)
Paper : Toyo-Roshi No. 51
Ascending method

Fig. 3. Paper electrophoresis of O-2867-α and -β
Buffer pH 2.0 : McILVAINE's buffer
3.7 : Pyridine-acetic acid-water (1 : 10 : 289)
8.0 : M/15 phosphate buffer
200 V, 2.5 hours

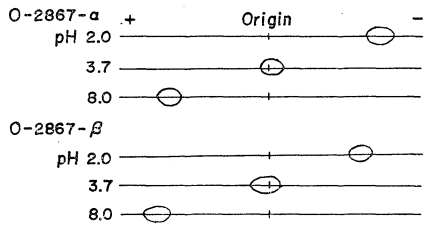
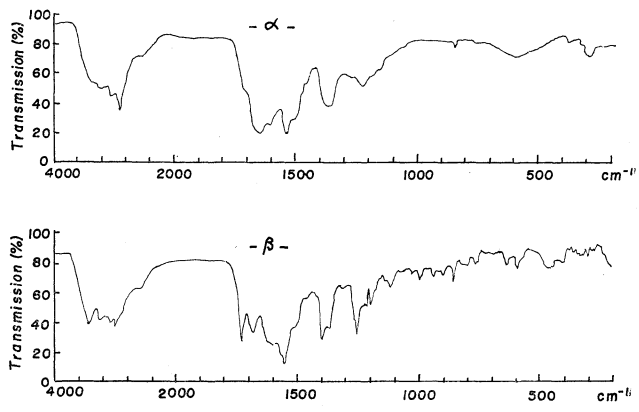


Fig. 4. Infrared absorption spectra (in KBr)



0.5 N hydrochloric acid and concentrating the eluate *in vacuo*. The powder thus obtained was chromatographed on a carbon column developed with a gradient of 0 to 25 % aqueous acetone. Two active fractions, α (the first eluted) and β, were detected by bioassay with *P. oryzae*. Each fraction was rechromatographed separately by the same procedure, then purified further on a column of cellulose powder developed with *n*-butyl alcohol - acetic acid - water (4 : 1 : 2). The active fractions were lyophilized to yield white powders. O-2867-β was crystallized and recrystallized from water as needles, but O-2867-α could be obtained only in an amorphous state. The isolaion scheme is shown in Fig. 2.

Physical and Chemical Properties

Physical and chemical properties of antibiotics O-2867-α and β are summarized in Table 1. Paper electrophoresis is shown in Fig. 3. This indicates that O-2867-α and β are amphoteric. The two antibiotics have similar solubilities and give similar functional group tests (Table 1). These suggest that a free amino or acid amide but no amino sugar group in present. This conclusion is supported by the infrared spectra (Fig. 4). Absorption maxima at 3600~2800 cm^{-1}, 1650 cm^{-1}, 1550 cm^{-1} and 1280 cm^{-1} can be characteristic of amines or acid amides[1]. Both antibiotics show only end absorption in the ultra violet spectrum.

Biological Properties

Table 2 shows the antimicrobial spectra of antibiotics O-2867-α and β. They are selectively active against *P. oryzae* and O-2867-α is also active against *Colletotrichum lagenarium* but they are not active against bacteria, yeasts and other fungi.

Table 2. Minimum inhibitory concentration (mcg/ml)

Test organisms	α	β
Staphylococcus aureus 209P*	>100	>100
Bacillus subtilis PCI 219*	>100	>100
Escherichia coli NIHJ*	>100	>100
*Pseudomonas aeruginosa**	>100	>100
*Shigella sonnei**	>100	>100
*Salmonella typhimurium**	>100	>100
*Proteus vulgaris**	>100	>100
Mycobacterium ATCC 607*	>100	>100
Xanthomonas oryzae	>100	>100
Candida albicans	>100	>100
Saccharomyces cerevisiae	>100	>100
Trichophyton roseum	>100	>100
Rhizopus sp.	>100	>100
Aspergillus niger	>100	>100
Penicillium notatum 4640	>100	>100
Gibberella saubinetii	100	>100
Neurospora crassa 4a	100	>100
Ophiobolus miyabeanus	>100	>100
Glomerella cigulata	>100	>100
Diaporthe nomserae Ni 2036	>100	>100
Sclerotinia cinerea	>100	>100
Pellicularia sasakii	>100	>100
Colletotrichum lagenarium	6.25	>100
Cephalosporium caerulens	>100	>100
Verticillium albo-atrum IFO 5922	>100	>100
Acrocylindrium oryzae 4130	>100	>100
Botrytis cryptoneriae	>100	>100
Piricularia oryzae KF 92	6.25	6.25
KF 93	12.5	6.25
KF 94	12.5	6.25
KF 96	12.5	3.13
KF 141	12.5	6.25
KF 180	12.5	12.5
IFO 5279	12.5	6.25
IFO 5994	25	6.25
Piricularia grisea IFO 5280	25	>100
Thielaviopsis basicola IFO 6116	>100	>100
Cladosporium wernecki	>100	>100
Coryneum carpophilum IFO 5908	>100	100
Brachysporium sp. 1	>100	>100
Alternaria kikuchiana	>100	>100
Cochliobolus miyabeanus	>100	>100
Fusarium moniliforme USDA 1004 1	>100	>100

Medium : rice straw extract agar * nutrient agar

Discussion

Of the known antifungal antibiotics blasticidin S[2], kasugamycin[3,4], and polyoxins[5,6,7,8,9] have been reported to be active against *P. oryzae*. The physico-chemical and biological properties of these antibiotics are different from those of O-2867-α and β. Sangivamycin[10] is similar to O-2867-α in being active against *P. oryzae* and *Colletotrichum* sp., but differs in its ultraviolet absorption spectrum. Some physico-chemical and biological properties of these antibiotics are compared in Table 3. Though our antibiotics are active against *P. oryzae in vitro*, they are ineffective in the preliminary pot test using rice plants infected with *P. oryzae*.

Table 3. Comparison of some physico-chemical and biological properties among antipiricularia antibiotic

Antibiotic	Nature	UV-Absorption (mμ)		Antimicrobial activity against
		Acid	Alkali	
Blasticidin S[2]	Basic	274	266	*Piricularia oryzae*, certain pseudomonas
Kasugamycin[3,4]	Basic	End		*Piricularia oryzae*, certain bacteria
Polyoxins[5,6,7,8,9]	Amphoteric	259～262	262～271	*Piricularia oryzae* certain phytopathogenic fungi
Sangivamycin[10]	Basic	233～278	234～282	*Piricularia oryzae* *Colletotrichum lindemuthianum*
O-2867-α	Amphoteric	End		*Piricularia oryzae* *Colletotrichum lagenarium*
O-2867-β	Amphoteric	End		*Piricularia oryzae*

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