A NEW ANTIBIOTIC, BULGERIN, ACTIVE AGAINST PHYTOPATHOGENIC FUNGI

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(Received for publication April 30, 1970)

A new antibiotic, bulgerin, was isolated from a culture broth of a streptomyces strain S-288. The strain was classified as *Streptomyces aburaviensis* var. *tuftformis*. The antibiotic is a water-soluble amphoteric substance, $C_{17}H_{24}O_{10}N_4$, λ_{max} 230 mμ (shoulder) and 289 mμ in 0.05 N HCl, $[\alpha]_D$ +24.0° in water, and is active against some phytopathogenic fungi.

In the course of our screening program, a streptomyces strain S-288 was found to produce an antibiotic which exhibits a bulging effect[1] against *Piricularia oryzae*.

This strain was isolated from a soil sample collected at Mie Prefecture, Japan. As a result of taxonomic studies by the usual methods, its characteristics are summarized as follows: no whorl, no spiral, smooth surface of spores, yellowish brown growth with brownish gray aerial mycelium and yellowish brown soluble pigment on various media, non-chromogenic type, proteolytic action with peptonization of milk, and liquefaction of gelatin. Among the known species of streptomyces having these characteristics, *Streptomyces aburaviensis* NISHIMURA *et al.*[2] and *Streptomyces aburaviensis* var. *ablastmyceticus* HAMADA *et* OKAMI[3] are most closely related to this strain. However, some differences are found among these strains as shown in Table 1. Therefore, the strain S-288 is designated as *Streptomyces aburaviensis* var. *tuftformis* var. nov. MAYAMA *et* KAWAMURA.

The antibiotic, named bulgerin, was isolated from the fermentation broth of the

Table 1. Comparison of *S. aburaviensis* var. *tuftformis* with related species

	S. aburaviensis var. *tuftformis*	*S. aburaviensis* [2]	*S. aburaviensis* var. *ablastmyceticus* [3]
Sporophore	Straight, tuft	Straight, irregular	Flexuous
Nitrate reduction	−	+	−
Utilization of carbohydrates			
mannose	++	−	Utilized to some extent
fructose	−	±	Well utilized
xylose	++	−	Well utilized
arabinose	++	−	Well utilized
sucrose	−	−	Well utilized
lactose	±	−	Not utilized
salicin	±	−	Not utilized

strain S-288 by ion-exchange resin procedure. It was purified by gel-filtration and preparative paper chromatography. Homogeneity was confirmed by paper chromatographic and thin-layer chromatographic examination : Toyo Roshi No. 51, *n*-butanol - acetic acid - water (4 : 1 : 2), Rf 0.20 and *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12), Rf 0.60 ; Silica gel GF, chloroform - methanol - 17 % aq. ammonia (2 : 1 : 1, upper layer), Rf 0.80 and *n*-butanol - ethanol - 0.1 N hydrochloric acid (1 : 1 : 1), Rf 0.50. An amphoteric nature with an isoelectric point between pH 5.0 and pH 6.0 was indicated by paper electrophoresis carried out using buffer solutions of various pHs.

Fig. 1. Ultraviolet absorption spectrum of bulgerin.

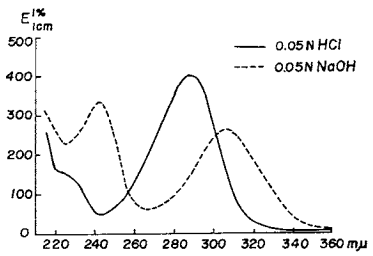
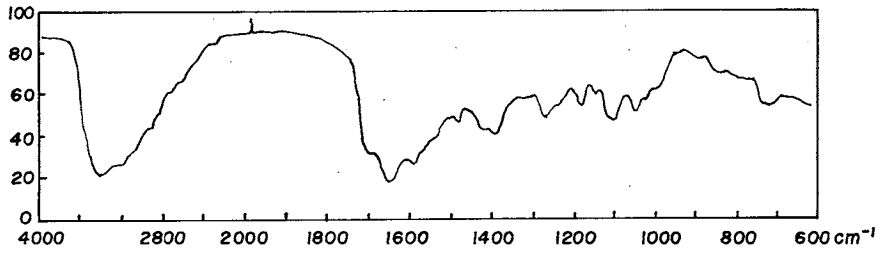


Fig. 2. Infrared absorption spectrum of bulgerin (KBr).



The free form of the antibiotic was obtained as a colorless amorphous powder. It has no definite melting point and decomposes gradually above *ca.* 170°C. It is optically active : $[\alpha]_D^{22}$ +24.0°±1.3° (*c* 0.490, H_2O). It exhibits the following maxima in its ultraviolet absorption spectrum as illustrated in Fig. 1 : $\lambda_{max}^{0.05\,N\,HCl}$: 230 mμ (shoulder) ($E_{1cm}^{1\%}$ 147), 289 mμ ($E_{1cm}^{1\%}$ 410), $\lambda_{max}^{0.05\,N\,NaOH}$: 242 mμ ($E_{1cm}^{1\%}$ 336), 306 mμ ($E_{1cm}^{1\%}$ 263). The infrared absorption spectrum measured on a KBr tablet is shown in Fig. 2. Elemental analysis and molecular weight determination by potentiometric titration indicate a molecular formula of $C_{17}H_{24}O_{10}N_4$.

Table 2. Antifungal spectrum of bulgerin

Test organism	MIC (mcg/ml)
Piricularia oryzae	1.6
Colletotrichum sp.	6.2
Gloeosporium kaki	>100
Corticium sasakii	6.2
Fusarium oxysporum	>100
Sclerotinia libertiana	>100
Cochliobolus miyabeanus	0.8
Alternaria kikuchiana	>100
Candida albicans	50
Aspergillus niger	>100
Trichophyton purpreum	>100
Trichophyton mentagrophytes	>100
Trichophyton rubrum	>100
Epidermophyton floccosum	>100

The antibiotic is readily soluble in water, but hardly soluble or insoluble in common organic solvents. It gives a positive reaction with ninhydrin and decolorizes potassium permanganate, but is negative with PAULY and DRAGENDORF reagents.

The antibiotic bulgerin is not active against bacteria so far as tested, but is active against some phytopathogenic fungi. The minimum inhibitory concentrations (MIC) are shown in Table 2.

Some antifungal antibiotics which have characteristic ultraviolet absorption indicative of the presence of purine or pyrimidine base-like constituents have been isolated

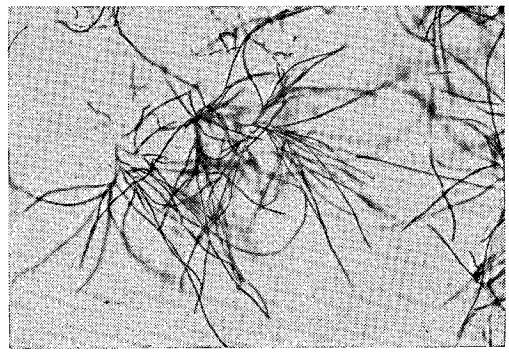
from streptomyces strains. Bugerin is considered to have some resemblance to polyoxins[4,5,6] and ablastmycin[3] in structural features. However, bulgerin differs in UV-maxima, indicating that it has a different chromophore, probably a purine or pyrimidine base-like component. On hydrolysis, bulgerin gives a UV-absorbing substance and ninhydrin-positive substances, both of which are recognized to be different from the hydrolysates of polyoxins A and B by direct comparison[8]. Formycin[7] has similar UV-maxima, but it is easily differentiated by other characteristics. Thus, bulgerin is considered to be a new antibiotic exhibiting antifungal activity.

Experimental

Taxonomic Studies:

I. Morphological characteristics: The culture was incubated on BENNETT's agar medium at 28°C for 14 days. An aerial mycelium were formed abunduntly and macroscopically showed a powdery to velvety type. The sporulating hyphae are straight to flexuous and branch in typical tuft form (Fig. 3). The spores are formed in chains with 10 to 50 spores, or sometimes more than 50 spores per chain. The shape of the spore is oval to cylindrical (0.6~0.8 μ in width, 1.0~1.2 μ in length). The surface of the spore is smooth. Sporangium and flagellated spores were not observed and also fragmentation and sclerotia in substrate mycelium were not observed.

Fig. 3. Aerial mycelium of strain S-288 on BENNETT's agar. (×200×1/1.5)



II. Cultural and physiological characteristics: Incubation was carried out at 28°C for 14 days.

1. On CZAPEK's agar: Yellowish gray growth; yellowish gray aerial mycelium; no soluble pigment.
2. On glucose-asparagine agar: Yellowish brown growth; light brownish gray aerial mycelium; no soluble pigment.
3. On glycerol-asparagine salt agar: Yellowish brown growth; yellowish gray aerial mycelium; no soluble pigment.
4. On calcium malate agar plate: Yellowish brown growth; brownish white to light brownish gray aerial mycelium; none to pale yellowish brown soluble pigment.
5. On glucose-CZAPEK solution: Light brownish gray to pale yellowish brown growth; no aerial mycelium; no soluble pigment.
6. On cellulose agar: Colorless growth; brownish white aerial mycelium; no soluble pigment.
7. On starch agar: Yellowish brown growth; light brownish gray aerial mycelium; no soluble pigment.
8. On nutrient agar: Yellowish brown growth; grayish white aerial mycelium; pale yellowish brown soluble pigment.
9. On glucose bouillon agar: Yellowish brown growth; white to grayish white aerial mycelium; pale yellowish brown soluble pigment.
10. On glucose peptone agar: Yellowish brown growth; white aerial mycelium; pale

yellowish brown soluble pigment.

11. On BENNETT's agar: Yellowish brown growth; brownish gray aerial mycelium; none to pale yellowish soluble pigment.
12. On potato plug: Yellowish brown growth; light brownish gray to light gray aerial mycelium; pale yellowish brown soluble pigment.
13. In milk medium: Pale yellowish brown growth; grayish white aerial mycelium; none to pale brown soluble pigment; peptonization occurs.
14. On gelatin medium; Light brown growth; no aerial mycelium; brown soluble pigment; relatively strong liquefaction of gelatin.
15. In glucose broth: Grayish yellow brown growth; white aerial mycelium; no soluble pigment.
    Growth type is ring.
16. Utilization of carbon sources on PRIDHAM-GOTTLIEB's basal agar medium: Growth was good on glycerol, mannose, xylose, arabinose, glucose, galactose, maltose, dextrin and starch; moderate to slight on lactose and salicin; no growth on mannitol, fructose, raffinose, sucrose, sorbitol, rhamnose, inulin, sorbose, inositol and dulcitol.

Production:

The streptomyces strain S-288 was used to inoculate on 800 ml of medium consisting of starch 1.0 %, soybean meal 1.0 %, corn steep liquor 0.5 % and NaCl 0.3 %, pH 7.0, in a 2-liter Erlenmyer flask, and was cultured at 27°C for 48 hours on rotary shaking machine. The culture was then transferred to a 30-liter jar fermentor containing 15 liters medium consisting of starch 2.0 %, soybean meal 2.0 %, corn steep liquor 0.5 % and $(NH_4)_2SO_4$ 0.2 %, pH 7.0. Fermentation was carried out at 28°C for 3 days under aeration of 20 liters per minute and agitation of 250 r.p.m.

Isolation and Purification:

Some 60 liters of the cultured broth obtained as above was filtered at pH 3.0 by filter-aid. The filtrate was further acidified and passed through a 5-liter column of Dowex 50 W (Na type). The adsorbed antibiotic was eluted with 0.3 N NH_4OH. Active eluates tested on a *P. oryzae* assay plate were combined, and adsorbed on 60 g of Darco G-60 at neutral pH. After washing with water, the carbon was eluted with 100 ml of 60 % aq. acetone, repeatedly. The eluate was partially evaporated under reduced pressure, then lyophilized to give a brown residue (approx. 6 g). The product was purified by gel filtration on a Sephadex G-10 column (2.5×90 cm) with 0.5 % acetic acid. Each run was carried out with approx. 2 g of the residue. Filtrates were tested by paper chromatography and rich fractions were lyophilized to yield 1.1 g of crude powder (approx. 500 mcg/mg).

Some 200 mg of the crude powder was applied to a chromatography paper (Toyo Roshi No. 525, 60×60 cm), and developed with *n*-butanol - acetic acid - water (4 : 1 : 2) by a descending and continuous developing method for 18 hours. The zone containing the antibiotic was detected by UV and ninhydrin reaction, and was extracted with aq. methanol. The eluate was freeze-dried. The preparative paper chromatography was similarly repeated with *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12), and finally, a biologically pure preparation of the antibiotic was obtained as a pale yellowish powder (yield: approx. 50 % in activity). For an analytically pure preparation, the above preparation was adsorbed onto Darco G-60 (5 volumes w/w)

which was pre-washed with hot water and acetone and dried. The carbon was washed with water and eluted with 60 % aq. acetone. Evaporation and lyophilization afforded a colorless amorphous powder.

Anal. Found : C 45.63, H 5.15, N 12.57.
Calcd for $C_{17}H_{24}O_{10}N_4$: C 45.94, H 5.44, N 12.61 %, MW 444.38.

Titration equivalents by hydrochloric acid in water and by perchloric acid in acetic acid were 409 and 149.5, respectively.

Acknowledgement

The authors express their sincere thanks to Dr. K. Isono, Institute of Physical and Chemical Research, for his kind gift of polyoxins A and B.

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