ABLASTMYCIN, A NEW ANTI-PIRICULARIA ANTIBIOTIC

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A new antibiotic, ablastmycin ($C_{18}H_{31}N_5O_{10}$, λ_{max} 274 mμ in 0.1 N HCl, $[\alpha]_D^{25}=+60°$ in H_2O), inhibiting *Piricularia oryzae* and *Helminthosporium* was isolated. The producing strain was classified as *Streptomyces aburaviensis* var. *ablastmyceticus*. Production, isolation, purification and physico-chemical properties of this antibiotic are described.

Kasugamycin which shows preventive effect on rice blast and low toxicity to plants and animals was discovered in a culture filtrate of Actinomycetes. The study on kasugamycin, as described in a previous paper[1], established a new screening method to examine the inhibition of *Piricularia oryzae* growing in an acidic medium prepared with rice plant juice. Applying this screening method, a new antibiotic designated ablastmycin was found in the culture filtrate of a streptomyces. This antibiotic inhibits *Piricularia oryzae in vitro* at very low concentration and has low phytotoxicity, though the protective effect is reduced remarkably in the green house test.

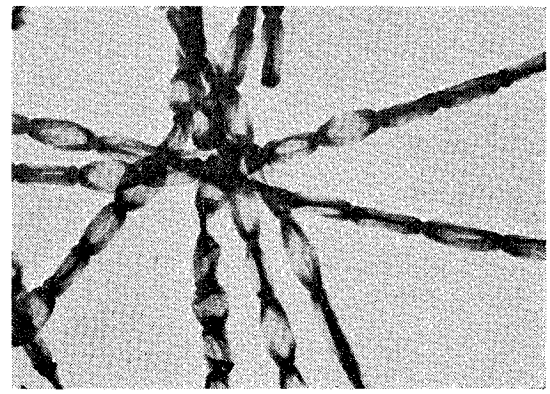
In this paper, the taxonomy of the streptomyces producing this antibiotic, the condition for optimum production, isolation and purification procedures, and the properties are reported.

Ablastmycin-Producing Organism

The culture was isolated from a soil sample collected at Utsukushi no Mori in Nagano Prefecture, Japan. It was isolated on glycerol-casein agar and designated M 190-C 3. Its characteristics on various media are as follows:

On a synthetic agar plate, long flexuous aerial mycelia develop from fine branched substrate mycelia, surface of spores is smooth under the electron microscope (Plate 1).

Plate 1. *S. aburaviensis* var. *ablastmyceticus* M 190-C 3 ×8,000



Characters on Various Media

1. On glycerol CZAPEK's agar (incubated at 27°C): Yellowish brown to dark brown growth; white to brownish gray aerial mycelium; yellow to dark yellowish brown colored soluble pigment.

2. On KRAINSKY's glucose asparagine agar (at 27°C): Reddish yellow to dark yellow growth; white to brownish gray aerial mycelium; dark yellow soluble pigment around growth; medium is colored slightly yellow.
3. On calcium malate agar plate (at 27°C): Pale yellowish brown growth; white to light gray aerial mycelium; no soluble pigment.
4. In peptone solution containing 1.0% sodium nitrate (at 27°C): Colorless growth; no aerial mycelium; no soluble pigment; negative reaction for nitrate reduction.
5. On potato plug (at 27°C): Pale yellowish brown to dark yellowish brown growth; white to brownish gray aerial mycelium; pale yellowish brown to dark yellowish brown soluble pigment.
6. On starch agar plate (at 27°C): Pale yellowish brown to pale brown growth; white to light brownish gray aerial mycelium; yellow to pale brown or pale orange soluble pigment; weak hydrolytic action on starch.
7. On nutrient agar (at 37°C): Pale yellowish brown to pale brown growth; no soluble pigment; no aerial mycelium.
8. On nutrient agar (incubated at 27°C): Same as incubated at 37°C.
9. On blood agar (at 37°C): Pale yellowish brown to grayish red brown growth; no aerial mycelium; practically negative hemolysis.
10. On LOEFFLER's coagulated serum medium (at 37°C): No growth.
11. On gelatin stab (at 20°C): Yellowish brown to light brown growth; thin, white to grayish white aerial mycelium; light brown soluble pigment; relatively strong liquefaction of gelatin.
12. In milk medium (at 37°C): Cream to pale yellowish brown; later yellowish brown or orange colored growth; no soluble pigment; no aerial mycelium; after completion of coagulation, relatively strong peptonization occurs.
13. On tyrosine agar (at 27°C): Colorless to pale yellowish brown growth; thin, white aerial mycelium; pale yellowish brown soluble pigment; negative tyrosinase reaction.
14. On cellulose (at 27°C): No growth.
15. Utilization of carbon sources for growth on PRIDHAM-GOTTLIEB's basal agar medium (incubated at 27°C): Glycerol, xylose, fructose, galactose, glucose, sucrose, dextrin and starch are well utilized; arabinose, maltose and mannose are utilized at some extent; rhamnose, inositol, mannitol, sorbitol, dulcitol, lactose, raffinose, salicin and inulin are not utilized.

Summarizing the above data, the strain M 190-C 3 belongs to the genus *Streptomyces*, and has the following characteristics, no whorl, no spiral, smooth surface of spore, yellowish brown growth with brownish gray aerial mycelium, and yellowish brown soluble pigment on various media, non-chromogenic type, fairly strong proteolytic action with peptonization of milk and liquefaction of gelatin.

Among the known species, the strain No. M 190-C 3 is most similar to *Streptomyces aburaviensis* NISHIMURA *et al.*[2] 1957, for which the electron microscopic picture of spore was reported[3]. It is noted, however, several differences are found between the strain M 190-C 3 and *S. aburaviensis* as shown in Table 1.

Table 1

	M190-C3	*S. aburaviensis*
Sporophore	Flexuous	Straight
Aerial mycelium	Brownish gray	White to faint grayish
Nitrate reduction	−	+
Utilization of carbohydrates		
sucrose	Utilized	Not utilized
xylose	Utilized	Not utilized
inulin	Not utilized	Utilized
Antibiotic produced	Ablastmycin	Aburamycin

Thus, the strain M 190-C 3 is assigned to a new species named

Streptomyces aburaviensis var. *ablastmyceticus* HAMADA *et* OKAMI.

Assay of Ablastmycin

Piricularia oryzae is incubated at 27°C for 7 days on a medium containing 1.0 % soluble starch and 0.2 % powdered agar. Then, the culture is incubated for 3 days at 20°C to give good sporulation.

From this sporulated culture, a spore suspension is prepared and used as the seed layer of the assay plate. The medium for both seed layer and base layer is prepared as follows; 100 g of fresh rice straw is boiled for 30 minutes in 1 L of water and filtered; to the filtrate of this extract 1.5 % sucrose and 2.2 % powdered agar are added, and it is then autoclaved. A 0.1 M citrate-phosphate buffer at pH 5.0 is prepared and sterilized; at the time of preparation of the assay plate, equal volumes of the medium and the buffer solution are mixed. The assay of ablastmycin is performed by the ordinary cylinder plate method for assay of antibiotics. Solutions of 1.0, 0.25 and 0.15 mcg/ml of ablastmycin show inhibition zones of 36.5, 25.5 and 22.0 mm in diameter after incubation at 27°C for 2 days.

Helminthosporium oryzae can also be employed for assay of ablastmycin. A spore suspension is prepared from a 10-day slant culture at 27°C on a medium containing of 3.0 % sucrose, 1.0 % KNO_3, 0.5 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.075 % dried yeast (Ebios) 2~3 drops of 1.0 % $FeCl_3$ solution and 1.7 % powdered agar (pH 6.0~6.2).

Production of Ablastmycin

Strain M 190-C 3 was inoculated into a 500-ml flask containing 125 ml of medium consisting 1.0 % glucose, 1.0 % starch, 1.5 % soybean meal, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$ and 0.3% NaCl. It was cultured at 27°C for 4 days on a reciprocating shaking machine (140 strokes/min. with amplitude of 8 cm). Production rate and pH changes in varied media are shown in Table 2.

In a jar fermenter, ablastmycin was produced as follows; the same medium used for the shaking culture was employed; 15 L of the medium was sterilized at 120°C for 30 minutes in a stainless steel fermenter of 30 L volume and fermented under

Table 2—1

	3rd day		4th day		5th day		6th day	
	pH	Production	pH	Production	pH	Production	pH	Production
Glucose 1.0% Starch 1.0 〃	4.6	0.93	6.8	2.80	7.6	4.56	8.6	1.68
Glucose 1.5 〃	4.6	0.44	7.2	0.93	8.2	0.53	8.4	0.35
Starch 1.5 〃	6.6	<0.1	6.0	0.47	7.0	0.93	8.2	0.67
Glycerol 1.5 〃	7.4	0.67	8.0	0.56	8.4	0.36	8.6	0.25
Maltose 1.5 〃	7.2	0.67	7.6	0.93	8.4	1.82	8.6	0.93
Dextrin 1.5 〃	6.6	0.44	6.2	1.75	7.2	2.20	8.2	2.46
Lactose 1.5 〃	6.4	0.36	7.0	0.56	7.6	0.93	7.8	0.81
Sucrose 1.5 〃	6.6	0.44	7.2	0.88	7.6	0.67	8.2	0.60

Except carbon sources, the medium contained 1.5 % soybean meal, 0.3 % NaCl, 0.1 % K_2HPO_4 and 0.05 % $MgSO_4 \cdot 7H_2O$ and the initial pH was adjusted to pH 7.0.

Table 2—2

	3rd day		4th day		5th day		6th day	
	pH	Production	pH	Production	pH	Production	pH	Production
Soybean meal 1.5% NaCl 0.3 〃 K_2HPO_4 0.1 〃 $MgSO_4 \cdot 7H_2O$ 0.05 〃	4.6	0.93	6.8	2.80	7.6	4.56	8.6	1.68
Peptone 0.75 〃 Beef ext. 0.75 〃 NaCl 0.3 〃	6.6	<0.1	7.4	<0.1	8.2	<0.1		
NZ-Amine 1.0 〃 Yeast ext. 0.3 〃 NaCl 0.3 〃	7.0	<0.1	8.0	<0.1	8.2	<0.1		

Each medium contained 1.0% glucose and 1.0% starch, as carbon sources.

aeration of 15 L air per minute and stirring at 250 r.p.m. at 27°C. Silicone resin (KM-66, Shinetsu Chemical Products) and soybean oil were employed as antifoaming agents.

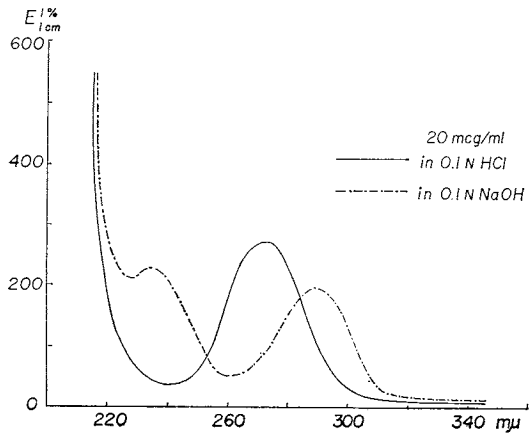
Fermented broths at the 2nd, 3rd, 4th and 5th days showed pH 5.4, 6.6, 7.4 and 8.2, and 10, 22, 22 and 15 mcg/ml of ablastmycin, respectively.

Isolation and Purification of Ablastmycin

Ablastmycin was not found in the mycelium mass, and was extracted from the fermentation liquid. The cultured broth was adjusted to pH 2.0 and filtered.

The filtrate was adjusted to pH 7.0 with 2 N NaOH and the resulting precipitate was filtered. The filtrate (26.5 L) was passed through a carbon column to adsorb the ablastmycin. After the column was washed with water and 0.1 N HCl, ablastmycin was eluted with 0.1 N HCl-acetone (1 : 1). Fractions which showed activity were combined, neutralized with Amberlite IR-45 (OH^-), and concentrated *in vacuo* to dryness. The dried powder was dissolved in 0.2 M pyridine-acetate buffer (pH 5.25), and chromatographed on Dowex 50 W-X 8 which had been treated with pyridine-acetate. The active fractions were combined and subjected to column chromatography on carbon. The column was washed with water and 0.1 N HCl, and ablastmycin was eluted with 0.1 N HCl-acetone (1 : 1).

Fig. 1. Ultraviolet spectra of ablastmycin
20 mcg/ml

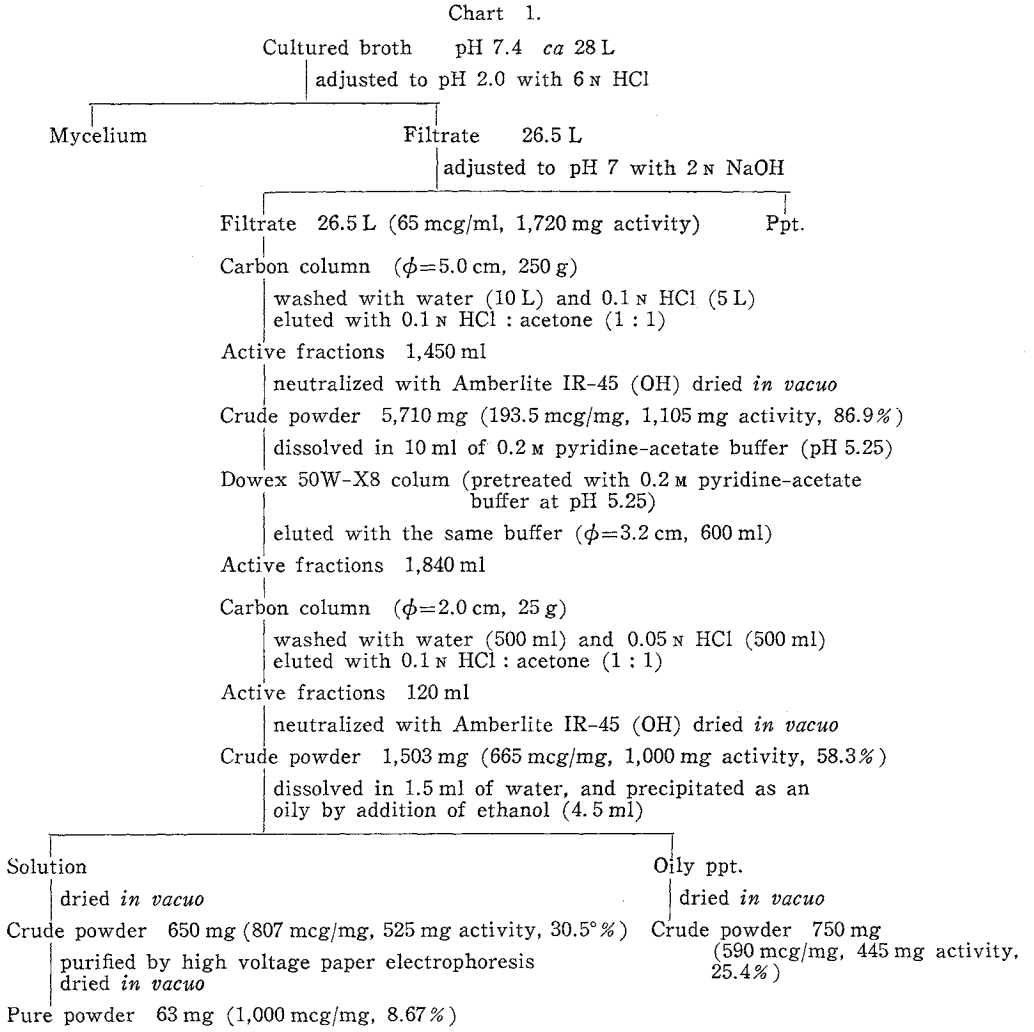


The active eluate was neutralized with IR-45 (OH^-), and concentrated *in vacuo* to dry powder. To the aqueous solution of this powder, absolute ethanol was added. The ethanol solution was heated and filtered to remove a small amount of insoluble solid. The filtered solution was kept at room temperature overnight to yield an oily material with part of the

activity, but most of the activity remained in the ethanol. The ethanol was evaporated *in vacuo* to a dry powder. This powder was subjected to high voltage paper electrophoresis at 3,000 V, 100~150 mA for 30 minutes using Whatmann No. 3 MM paper, and HCOOH : CH_3COOH : H_2O (25 : 75 : 900). One of the two ninhydrin-positive bands showed ultraviolet absorption and biological activity.

The active band was cut out, and extracted with water to yield 63.0 mg of pure ablastmycin. Procedure and yield are shown in Chart 1.

Chart 1.

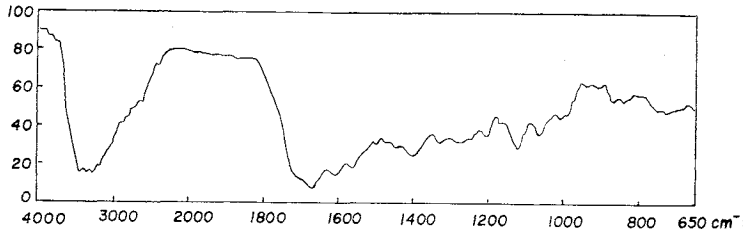


Properties of Ablastmycin

As shown in Fig. 1, ablastmycin has maxima at 274 mμ ($E^{1\%}_{1cm}$ 270) in 0.1 N HCl, and at 235 mμ ($E^{1\%}_{1cm}$ 225) and 290 mμ ($E^{1\%}_{1cm}$ 185) in 0.1 N NaOH. Infrared spectrum of ablastmycin is shown in Fig. 2.

Ablastmycin shows positive MOLISCH, anthrone, BIAL and ninhydrin (with pyridine) reactions. ELSON-MORGAN, sodium nitroprusside, MILLON, HOPKINS-COLE, xantho-protein, EHRLICH and ninhydrin (without pyridine) reactions are negative. Ablastmycin

Fig. 2. Infrared spectrum of ablastmycin



gives doubtful results with SELIWANOFF and TAUBER reactions.

Ablastmycin melts at 163~165°C (dec.), $[\alpha]_D^{25}+60°$ (c=0.5, H_2O). Molecular weight estimated by the titration equivalent and measured by vapor pressure osmometer using water as solvent is 242 and 482.5±2.5 respectively.

Anal. Found : C 44.72, H 6.40, N 14.60, O 32.96.
Cacld. for $C_{18}H_{31}N_5O_{10}$ (m. w. 479.48) : C 45.09, H 6.94, N 14.61, O 33.37.

Biological Activity of Ablastmycin

Ablastmycin inhibits *Piricularia oryzae* and *Helminthosporium oryzae* at 0.05 mcg/ml on a medium containing rice straw juice either at acidic or neutral pH, but it does not inhibit *Xanthomonas oryzae* or other microorganisms including plant pathogens.

As shown in Tables 3 and 4, ablastmycin shows no inhibition of microorganisms when tested on 1% glucose nutrient agar. Ablastmycin, though it is very active against *Piricularia* and *Helminthosporium in vitro*, did not show a protective effect against rice blast in the doses at which kasugamycin exhibits a preventive effect.

Table 3. Antimicrobial spectrum of ablastmycin (I)

Test organism	M.I.C. mcg/ml
Staphylococcus aureus FDA 209-P	>100
Staphylococcus aureus Terajima	>100
Sarcina lutea PCI 1001	>100
Micrococcus flavus 16	>100
Candida albicans 3147	6.25
Bacillus anthracis	>100
Escherichia coli NIHJ	>100
Shigella flexneri 1a Ew 8	>100
Salmonella enteritidis	>100
Pseudomonas aeruginosa A3	>100
Proteus vulgaris OX-19	>100
Klebsiella pneumoniae PCI 602	>100
Serratia marcescens	>100
Bacillus subtilis NRRL B-558	>100
Mycobacterium tuberculosis 607*	>100
*Mycobacterium phlei**	>100

Minimum inhibitory concentration of ablastmycin by agar streak method, using nutrient agar.
* 1.0% glycerol nutrient agar.

Table 4. Antimicrobial spectrum of ablastmycin (II)

Test organism	M.I.C. mcg/ml
Gibberella saubinetii	50
Helminthosporium oryzae (spore suspension)	12.5~50
Aspergillus niger	50
Trichophyton asteroides 429	50
Fusarium lini	50
Torula utilis 4001	50
Saccharomyces cerevisiae	50
Pseudomonas solanacearum	50
Pseudomonas fluorescens	50
Piricularia oryzae (spore suspension)	50
Xanthomonas oryzae (spore suspension)	50
Candida albicans Yu-1200	50
Candida krusei	50
Gloeosporirm kaki	50
Alternaria kikuchiana Tanaka	50
Candida tropicalis NI 7495	50
Pellicularia filamentosa Sasaki	50

Minimum inhibitory concentration of ablastmycin by agar streak method, using 1% glucose nutrient agar.

Differentiation of Ablastmycin from Other Anti-Piricularia Antibiotics

Ablastmycin can be differentiated from other anti-piricularia substances such as blasticidin S[4], kasugamycin, and polyoxins A and B[5] by the ultraviolet absorption spectrum and melting point as shown in Table 5. Polyoxins A and B[5] resemble ablastmycin in extraction procedure, but they are clearly differentiated not only by difference in the ultraviolet absorption and melting point, but also by difference in behavior on paper chromatography using a solvent, *n*-propanol : pyridine : acetic acid : water (15 : 10 : 3 : 12 v/v), by descending method for 18 hours. Ablastmycin moves 23 cm from the origin and polyoxins A and B move 18.5 and 14.5 cm, respectively.

Ablastmycin is thus considered to be a new antibiotic.

Table 5. Differentiation from other antibiotics

	Ablastmycin	Blasticidin S	Kasugamycin	Polyoxin A, B
U.V. max. 0.1 N HCl U.V. max. 0.1 N NaOH	274 mμ 235, 290 mμ	275 mμ 266~270 mμ		262 mμ 264 mμ
Optical rotation	+60° (H_2O)	+108.4° (H_2O)	+120° (H_2O)	A −32° (H_2O) B +34° (H_2O)
Melting point	163°C (dec.)	235~236°C	202~204°C (HCl)	

Discussion

Kasugamycin is a water-soluble basic substance, and effective in inhibiting *Piricularia* either *in vitro* or *in vivo* (in rice plant). It was also noted by UMEZAWA *et al.* that kasugamycin was inactive on *Piricularia in vitro* when the pH of the medium was not acidic. Ablastmycin is also a water-soluble basic substance, but it is active *in vitro* either at acidic or at neutral pH. It is not effective in inhibiting *Piricularia oryzae* in the rice plant, although it shows inhibition *in vitro* at very low concentration on a medium with added rice juice. There may be several reasons, why ablastmycin is not effective *in vivo* (rice plant). Ablastmycin has considerable strong ultraviolet absorption and therefore it may decompose when it is applied under sun light. It may diffuse into the rice plant. Also it may be inactivated in the rice plant. Ablastmycin shows no inhibition of *Piricularia oryzae* if its effect is tested in glycerol nutrient agar. It will be interesting to elucidate the reasons why ablastmycin is not active *in vivo*.

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