HERBIMYCIN, A NEW ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES*

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Herbimycin, a new antibiotic, was isolated from the fermentation broth of *Streptomyces hygroscopicus* strain No. AM-3672, a soil isolate. The molecular formula of herbimycin was determined to be $C_{30}H_{42}N_2O_9$. Herbimycin was found to have potent herbicidal activity against most mono- and di-cotyledonous plants, especially against *Cyperus microiria* STEUD. However, *Oryza sativa* showed strong resistance to herbimycin.

In the course of a screening program for herbicidal antibiotics of actinomycetes origin, we found a new antibiotic from the culture broth of *Streptomyces* sp. No. AM-3672. This strain was isolated from a soil sample collected at Kasumi-cho, Kinosaki-Gun, Hyōgo, Japan and identified as *Streptomyces hygroscopicus* WAKSMAN *et* HENRICI 1948. The UV, IR and NMR spectra suggested that the antibiotic belongs to the ansamycin group. It was named herbimycin because of its biological activity.

The present paper deals with the taxonomy of the producing strain and with the production, isolation and physicochemical as well as biological properties of the new herbicidal antibiotic.

Taxonomic Studies

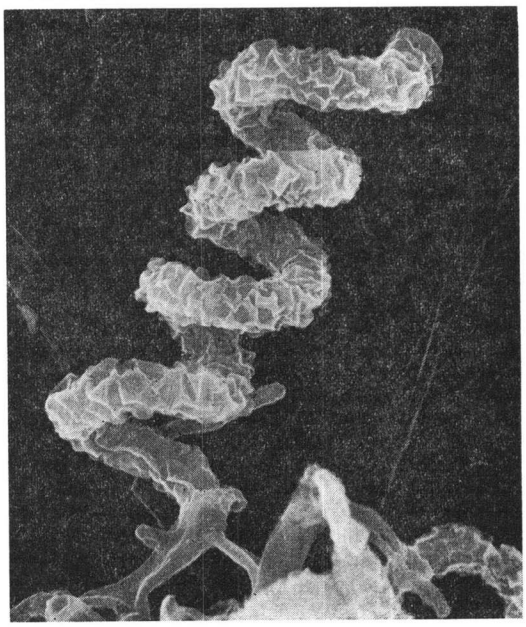
Morphological characteristics of strain AM-3672 were observed using cultures incubated at 27°C for 14 days on media such as oatmeal agar and glycerol-asparagine agar. The morphology was examined with both optical and electron microscopes. The aerial mycelia were simply branched and the chains of spores formed narrow compact spirals, but at an early age some of the chains were observed as open spirals. Sclerotic granules were not observed. Fig. 1 shows an electronmicrograph of conidia of strain AM-3672 cultured on oatmeal agar for 14 days. Conidia with warty surfaces were not segmented and sometimes showed a short cylindrical form ($0.7 \times 0.75\ \mu$). Cultural characteristics are shown in Table 1. The physiological properties and utilization of carbon sources are summarized in Tables 2 and 3, respectively. All cultures were incubated at 27°C for 21 days and observed every 7 days after incubation. The color terms recorded for each culture were described according to Color Harmony Manual[1].

LL-Diaminopimelic acid was found as a component of the cell wall of strain AM-3672. This and the

morphological characteristics described above indicate that the organism belongs to the genus *Streptomyces*. Its properties are summarized as follows: Genus, *Streptomyces*; spore chains, spirals with warty surface; aerial mycelium, hygroscopic properties; aerial mass color, white to brownish gray; melanoid pigment, none.

Among the known species of *Streptomyces* described in "BERGEY's Manual of Determinative Bacteriology" 8th Ed.[2] and WAKSMAN's "The Actinomycetes" Vol. II[3], *Streptomyces hygroscopicus* WAKSMAN *et* HENRICI, 1948 is similar to strain AM-3672. Since the morphological and cultural characteristics and the physiological properties of both strains are closely matched, strain AM-3672 is identified as a strain of *Streptomyces hygroscopicus*. It has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as *Streptomyces hygroscopicus* AM-3672 with the accession number FERM-P 4335.

Fig. 1. Electronmicrograph of the conidia of strain AM-3672.



Production and Isolation

A stock culture of *S. hygroscopicus* AM-3672 was used to inoculate 100 ml of medium in a 500-ml flask and incubated at 30°C on a rotary shaker (240 rpm). A 72-hour culture (300 ml) was transferred into 15 liters of medium in a 30-liter jar fermentor and the fermentation was carried out for 67 hours under the following conditions: Temperature, 30°C; aeration, 15 liters/min.; agitation, 300 rpm; pressure, 0.5 kg/cm^2; antifoam agent, Disfoam BC-51Y (Nihon Yushi-Chemical Co., Ltd.). The composition of seed and production medium was 2.5% glucose, 1.0% soybean meal, 0.4% KCl, 0.25% yeast extract, 0.1% meat extract, 0.5% $(NH_4)_2SO_4$, 0.02% K_2HPO_4 and 0.3% $CaCO_3$ (pH 7.2 before sterilization). Herbimycin was detected by thin-layer chromatography (TLC) on silica gel (Merck, GF_{254}), eluted with ethyl acetate - *n*-hexane - chloroform - methanol (9: 6: 1: 1, v/v); it was visualized by irradiation with UV light and its Rf value was 0.65.

A 67-hour culture (15 liters) of *S. hygroscopicus* strain AM-3672 was clarified with a Sharples centrifuge to obtain 12 liters of culture supernatant. This was used as starting material for the isolation of herbimycin. The supernatant was concentrated *in vacuo* to 2.5 liters. After the concentrate had been adjusted to pH 3.0 with 3 N hydrochloric acid, the antibiotic was extracted with two portions (2.5 liters each) of ethyl acetate. The extract was concentrated to 1.5 liters, washed twice with the same volume of 5% sodium bicarbonate solution and rewashed with 1 liter of saturated sodium carbonate solution. The solvent was removed under reduced pressure to give a yellowish brown paste. A 300-ml methanolic solution of the paste was mixed with 7 g of activated carbon. After the carbon cake had been washed with methanol, the adsorbed antibiotic was eluted with 700 ml of ethyl acetate. The eluate was concentrated under reduced pressure to give a crop of crude crystals. These were recrystallized from ethyl acetate and then from methanol to afford yellow columns

Table 1. Cultural characteristics of strain AM-3672.

Medium	Cultural characteristics
Sucrose-nitrate agar	G : moderate, cream (1½ ca) or pearl (ngs. 3ba) R : covert gray (ngs. 2fe) center is light ivory (2ca) AM : moderate, velvety, moist, white to covert gray (ngs. 2fe) SP : none
Glucose-nitrate agar	G : good, light maize (2ea) or light ivory (2ca) R : light ivory (2ca) center is honey gold (2ic) AM : moderate, velvety, white SP : none
Glycerol-asparagine agar (ISP)*	G : good, cream (1½ ca) to colonial yellow (2ga) R : outer, shadow gray (ngs. 5ih); intermediate, honey gold (2ic); center, cream (1½ ca) AM : abundant, velvety, moist, pearl gray (ngs. 13dc) sometimes white SP : none
Glucose-asparagine agar	G : good, cream (1½ ca) to ivory (2ca) R : outer, shadow gray (ngs. 5ih); intermediate, pearl (ngs. 2ba); center, melon yellow (3ga) AM : abundant, velvety, moist, shadow gray (ngs. 5ih) partially white SP : none
Glycerol-calcium malate agar	G : Good, cream (1½ ca) to light melon yellow (3ea) R : bamboo (2gc), center is butter yellow (1½ ga) AM : abundant, velvety, moist, white to beige gray (ngs. 3ih) SP : none
Inorganic salts-starch agar (ISP)*	G : good, light maize (2ea) to light ivory (2ca) R : outer, shadow gray (ngs. 5ih); intermediate, pearl (ngs. 2ba); center, honey gold (2ic) AM : abundant, velvety, moist, white to shadow gray (ngs. 5ih) SP : none
Tyrosine agar (ISP)*	G : good, light melon yellow (3ea) to light tan (3gc) R : bamboo (2gc) center is mustard brown (2pi) AM : abundant, velvety, moist, white to shadow gray (ngs. 5ih) SP : at first, flesh pink (4ca); later, none
Glucose-peptone agar	G : moderate, cream (1½ ca) or light ivory (2ca) R : light amber (3ic) or light maize (2ea) AM : moderate, velvety, moist, white SP : trace of light melon yellow (3ea)
Yeast extract-malt extract agar (ISP)*	G : good, cream (1½ ca) R : honey gold (2ic) AM : abundant, velvety, moist, cream (1½ ca) and white: sometimes partially beige gray (3ih) SP : none
Oatmeal agar (ISP)*	G : good, light maize (2ea) R : light ivory (2ca) partially covert tan (2ge) AM : abundant, velvety, moist, shadow gray (ngs. 5ih) SP : at first cream (1½ ca) later none
Peptone-yeast extract iron agar (ISP)*	G : good, light maize (2ea) R : topaz (3ne) or light ivory (2ca) AM : moderate, velvety, moist, light amber (3ic) or white SP : none
Nutrient agar	G : moderate, light ivory (2ca) R : light maize (2ea) or light ivory (2ca) AM : moderate, velvety, white SP : none

Abbreviation used in Table: G, growth; R, reverse; AM, aerial mycelium; SP, soluble pigment.

* Medium employed by International Streptomyces Project.

Table 2. Physiological properties of strain AM-3672.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	−
Hydrolysis of starch	+ 29.5/16.3*
Liquefaction of gelatin	+ strong (at 20°C)
Peptonization of milk	+
Coagulation of milk	±
Cellulolytic activity	−

* diameter of hydrolyzed area/diameter of colony

Table 3. Utilization of carbon sources by strain AM-3672.

Response	Carbon source
Positive	D-glucose, D-fructose, raffinose, *i*-inositol, rhamnose, D-mannitol
Doubtful	L-arabinose, sucrose, D-xylose
Negative	cellulose

(660 mg) of herbimycin.

Physicochemical Properties

Herbimycin is a neutral compound melting at 230°C and its optical rotation showed $[\alpha]_D^{20}$ +137° (*c* 1.0, chloroform). Elemental analysis gave the following values: C 62.82, H 7.35, N 4.88 (%). The calculated values for $C_{30}H_{42}N_2O_9$ are C 62.72, H 7.32, N 4.88 (%). The molecular formula determined from this analysis is supported by an accurate mass measurement of the molecular ion in its mass spectrum (Found, 574.295; Calcd. for $C_{30}H_{42}N_2O_9$, 574.289). The UV spectrum in methanol (Fig. 2) showed maxima at 270 nm (ε, 20,090) and 392.5 nm (ε, 1,650), which suggested the presence of a benzoquinone moiety[4]. The IR spectrum in chloroform (Fig. 3) showed the presence

Fig. 2. UV spectrum of herbimycin.

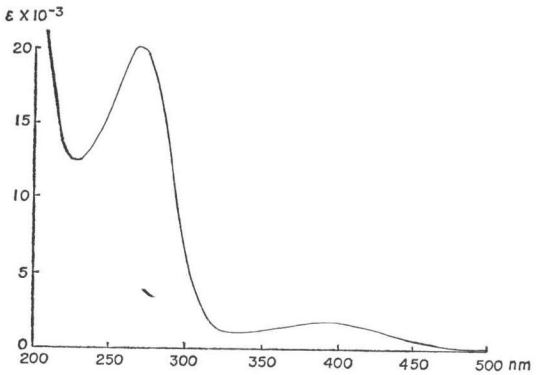
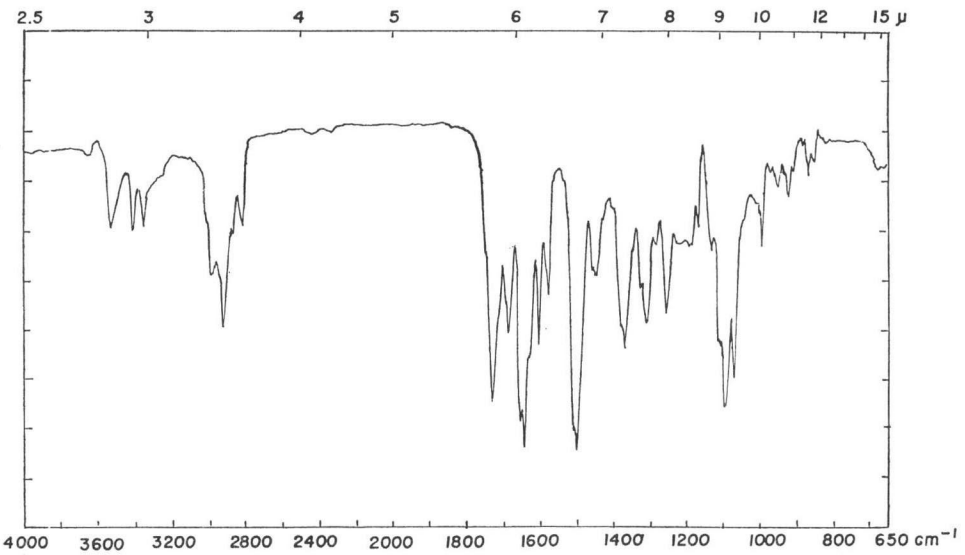


Fig. 3. IR spectrum of herbimycin.



of secondary or primary amine (3530, 3410, 3350 cm^{-1}), carbonyl (1730, 1690, 1655 cm^{-1}), double bond (1645, 1600 cm^{-1}) and ether (1095, 1070 cm^{-1}) groups. The mass spectrum of herbimycin showed a large peak at 531 in addition to weak peaks at 574 and 576. Accurate mass measurements indicated that the fragment peak at 531 corresponds to $C_{29}H_{41}NO_8$[M^+-43(HCON)]. Since some quinones give M^++2 peaks in the ion source of the mass spectrometer[5,6], the 576 peak should correspond to $C_{30}H_{44}N_2O_9$ (M^++2). The PMR spectra ($CDCl_3$, 100 MHz) and proton noise-decoupled ^{13}C-NMR ($CDCl_3$) are shown in Fig. 4 and Fig. 5, respectively. Herbimycin is soluble in acetone, chloroform, ethyl acetate, dimethylsulfoxide, N,N-dimethylformamide and, to a lesser extent, methanol, ethanol and benzene, but it is insoluble in water, ethyl ether and *n*-hexane. The physicochemical properties described above, especially the UV and NMR spectra, suggest that herbimycin belongs to the ansamycin antibiotics.

Fig. 4. PMR spectrum of herbimycin (in $CDCl_3$).

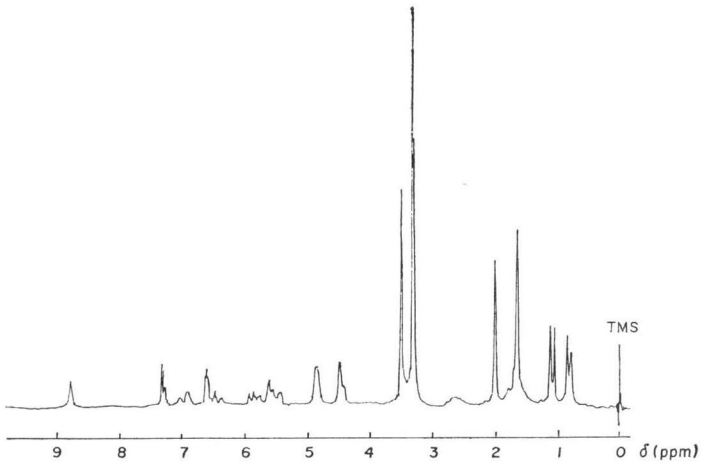
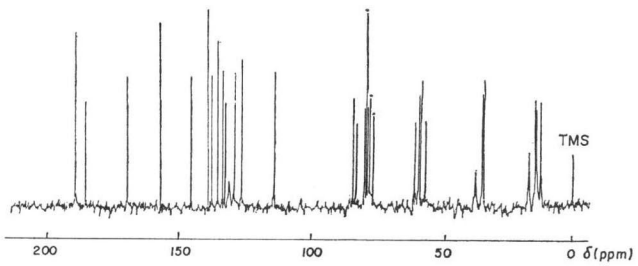


Fig. 5. ^{13}C-NMR spectrum of herbimycin (in $CDCl_3$).



Biological Properties

The antimicrobial spectrum of herbimycin was determined by the conventional agar dilution method using nutrient agar for bacteria (37°C, 24 hours) and glucose-potato agar for yeast and fungi (27°C, 72 hours). Herbimycin has weak activity against *Sarcina lutea, Candida albicans, Saccharomyces sake, Piricularia oryzae* and *Aspergillus niger* as shown in Table 4.

Table 4. Antimicrobial activity of herbimycin.

Test organism	Minimal inhibitory concentration (μg/ml)
Staphylococcus aureus FDA 209P	>200
Bacillus subtilis PCI 219	>200
Sarcina lutea PCI 1001	200
Mycobacterium smegmatis ATCC 607	>200
Escherichia coli NIHJ	>200
Salmonella typhimurium	>200
Pseudomonas aeruginosa P-3	>200
Candida albicans	200
Saccharomyces sake	200
Piricularia oryzae	200
Aspergillus niger	200
Microsporum gypseum	>200
Trychophyton interdigitale	>200
Trychophyton rubrum	>200

The herbicidal activity of herbimycin was tested by the following method. Plastic pots stuffed

Table 5. Herbicical activity of herbimycin (Pre-emergence system).

		Herbimycin (g/are)			
		100	50	25	12.5
Monocotyledon	*Oryza sativa*	2	1	1	0
	Echinochloa crus-galli	5	5	5	4
	Digitaria adscendens	5	5	5	4
	Cyperus microiria STEUD	5	5	5	5
Dicotyledon	*Chenopodium ficifolium*	5	5	5	4
	Postulaca oleracea	5	5	5	4
	Galinsoga ciliata	5	5	5	4
	Rorippa atrovirens	5	5	4	4

Table 6. Herbicidal activity of herbimycin (Post-emergence system).

		Herbimycin (g/are)		
		100	50	25
Monocotyledon	*Oryza sativa*	0	0	0
	Digitaria adscendens	5	4	4
	Cyperus microiria STEUD	5	5	5
Dicotyledon	*Chenopodium ficifolium*	5	5	5
	Postulaca oleracea	4	4	3
	Rorippa atrovirens	5	4	3
	Galinsoga ciliata	4	3	2

with sterile soil were placed in a greenhouse, planted with the seeds of various monocotyledonous and dicotyledonous plants, and then covered with soil to a depth of 5~10 mm. Two systems were used in treatments with herbimycin. The pre-emergence system (Table 5) was conducted before germination of the seeds and the post-emergence system (Table 6) was conducted two weeks after germination by foliar spraying. Herbicidal effects were examined on the 15th or 20th day after treatment with herbimycin and evaluated on a 0~5 scale as follows: 0, no activity; 1, <20%; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, 90~100%. As shown in Tables 5 and 6, herbimycin was found to have potent herbicidal activity against most of mono- and di-cotyledonous plants, especially against *Cyperus microiria* STEUD. However, *Oryza sativa* showed strong resistance. With most plants herbimycin was more active in the pre-emergence system than in the post-emergence system.

The acute toxicity (LD_{50}) of herbimycin in mice is approximately 19 mg/kg by intraperitoneal injection.

Discussion

In the course of the screening program for herbicidal antibiotics, herbimycin was isolated from the fermentation broth of *Streptomyces hygroscopicus* No. AM-3672, a soil isolate. Herbimycin has potent herbicidal activity and is selective in its killing effect. There is a difference between *Oryza sativa* and other plants such as *Cyperus microiria* STEUD. No antimicrobial activity was observed at 100 μg/ml concentration.

Among known antibiotics with an elemental analysis near that of herbimycin are geldanamycin[6],

mimosamycin[9] and kuwaitimycin.[10] However, none of their physicochemical properties are identical to those of herbimycin. The physicochemical properties, especially the molecular formula ($C_{29}H_{40}N_2O_9$) and IR spectrum of geldanamycin, the first ansamycin containing a benzoquinone nucleus, were found to resemble those of herbimycin. In addition, from the similar antimicrobial activity of the two antibiotics herbimycin was expected to be an ansamycin. Among known antibiotics, glutarimide antibiotics such as cycloheximide[11], nucleoside antibiotics such as herbicidin[7,8] and toyocamycin[12], and anisomycin[12] are reported to have herbicidal activity. However, our finding is new in that no ansamycin antibiotic has previously been reported to have herbicidal activity.

Acknowledgement

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