HERBICIDINS A AND B, TWO NEW ANTIBIOTICS WITH HERBICIDAL ACTIVITY

I. PRODUCING ORGANISM AND BIOLOGICAL ACTIVITIES

MAMORU ARAI, TATSUO HANEISHI, NOBUAKI KITAHARA,
RYUZO ENOKITA, *KATSUHIKO KAWAKUBO and *YASUHIKO KONDO

Fermentation Research Laboratories
*Agricultural Chemicals Research Laboratories
Sankyo Co., Ltd., 2–58, 1-chome, Hiromachi, Shinagawa-ku, Tokyo, Japan

(Received for publication June 23, 1976)

Herbicidins A and B, two new antibiotics with selective and contact herbicidal activity, were produced by a new species of *Streptomyces* designated as *S. saganonensis*. Among tested microorganisms, herbicidins indicated some activity against fungi *in vitro* and *Xanthomonas oryzae in vivo*. Their characteristic features were brought into relief by their selective and contact killing effect on many dicotyledonous plant as well as their inhibition of germination of the plant seeds, such as Chinese cabbage.

Only a few antibiotics, such as glutarimide-group antibiotics[1] and anisomycin and toyocamycin,[2,3] are known to have herbicidal activity. The former group exhibits contact but non-selective killing effect and the latter inhibit germination of various plant seeds.

In the course of an investigation directed toward the discovery of new antibiotics with herbicidal activity, we have also detected many strains of *Streptomyces* producing glutarimide antibiotics, but only one isolate of *Streptomyces,* strain No. 4075, isolated from a soil sample collected at Sagano, Kyoto Prefecture, was found to be capable of producing in its culture filtrate a herbicidal principle apparently different from known herbicidal antibiotics mentioned above.

The present paper describes taxonomic studies on the producing organism and biological properties of these new antibiotics.

Materials and Methods

Taxonomic studies. The cultural characteristics of the microorganism which produce herbicidins A and B were determined by the use of the media and methods described by SHIRLING and GOTTLIEB.[4] Observations of the culture were made after incubation at 28°C for two weeks, except where otherwise mentioned. The taxonomic key of BERGEY's Manual (8th ed.) and of WAKSMAN in The Actinomycetes, vol. 2 were used to the compare cultures with recognized genera and species of the actinomycetes.

Antibiotic samples. For the primary screening, the culture filtrate obtained by fermentation of strain No. 4075 in 500 ml Sakaguchi flask at 27°C for 6 days was diluted with water and used for the test of herbicidal activity. Even 30-fold dilution was effective in such trials. The medium for the production contained glycerol 2.0%, soybean meal 1.0%, corn-steep liquor 1.0%, $CaCO_3$ 0.3% and KH_2PO_4 1.0% (pH 7.2, before sterilization). Herbicidins were purified on Duolite S–30 column followed by counter-current distribution and silica gel column but details of the isolation procedure as well as fermentation for large scale production will be reported in the subsequent paper.

Biological activities.

(1) Antimicrobial activity. The minimal inhibitory concentrations (MIC) of herbicidins against

bacteria, yeasts and fungi were determined by conventional two-fold agar-dilution method. The medium used for bacteria was 1% glycerol-nutrient agar, for yeasts and fungi, such as *Trichophyton, Aspergillus* and *Penicillium,* was SABOURAUD dextrose agar and for other fungi was potato-sucrose agar. The MIC were examined after incubation of the test organisms for 2 days at 37°C for bacteria and for 2~14 days at 26°C for yeasts and fungi.

(2) Protection from bacterial leaf blight. Young rice plants (variety Kinmaze) having 5~6 leaves were sprayed with the sample solution to be tested (10 ml per pot), dried in the air and inoculated with a suspension of bacterial pathogen for leaf blight, *Xanthomonas oryzae* H 5809, previously grown on an agar slant composed of sucrose 1.5%, $Ca(NO_3)_2$ 0.05%, peptone 0.5% and Na_2HPO_4 0.2% in potato-extract agar. Cultivation at 28~30°C for 24 hours was performed in the room with 100% relative humidity and then at 28°C for 14 days in the greenhouse. All of the leaves were examined for the presence or absence of disease to calculate the percentage of the diseased leaves.

(3) Inhibitory effect on plant-seed germination. Eight seeds of Chinese cabbage or rice plant were placed on the absorbent cotton bed (10 mm height from the bottom of 18×200 mm test tube) immersed with 1.5 ml of the sample solution or with distilled water as blank. Minimal inhibitory concentration at two-fold dilution was determined by inspection of the lowest concentration required for the inhibition of germination of all of the seeds after incubation of the test tubes at 28°C for 3 days.

(4) Herbicidal activity. Unglazed flowerpots or plastic pots stuffed with soil were planted with various monocotyledonous and dicotyledonous plants and covered with soil to a depth of 5 or 10 mm. The pots were placed in a greenhouse for two weeks to allow the plant growth and then the sample solutions to be tested were directly sprayed over the leaves and stems of the plants. Herbicidal effect was examined on the 10th or 15th day after application of the samples and scored the grade of effectiveness in a manner as follows: The score is 0 when damaged area of the stems and leaves occupies 0~5% in comparison with that of untreated control, 1 for 5~20%, 2 for 20~50%, 3 for 50~80%, 4 for 80~95% and 5 for 95~100%.

Results and Discussion

Taxonomic Studies

The herbicidin-producing organism, isolate No. 4075, was classified as a member of the genus *Streptomyces.* The aerial hyphae indicated monopodial branching with sporophores of Rectus-Flexibilis on most of the media tested, Spira occurred on some media such as sucrose-nitrate agar. The spores were oval in shape, 0.4~0.5×0.6~1.0 μ in size and with smooth to warty surfaces. Photographs of sporulating hyphae and electron micrograph of spore chains of isolate No. 4075 are shown in Plates 1 to 4.

The cultural characteristics of isolate No. 4075 are summarized in Table 1. On most of the media the color of the substrate mycelium was white to grayish olive and the mass color of the aerial mycelia was white to yellowish gray. Physiological properties and utilization of carbon sources are shown in Tables 2 and 3. After comparison of these characteristics with those of the known species of *Streptomyces, S. fluorescens*[5] was selected as the most closely related one.

As shown in Tables 2 and 4, however, differences between isolate No. 4075 and *S. fluorescens* were obvious from the data described in the literature, coupled with the facts obtained by simultaneous comparison of the cultures of isolate No. 4075 with *S. fluorescens* ISP 5203. On the basis of these discrepancy, we propose that the culture be assigned a new species with the name *Streptomyces saganonensis* sp. nov. ENOKITA *et* ARAI.

Biological Activities

(1) Antimicrobial activity.

Plate 1. Photograph of aerial hyphae of isolate No. 4075, showing RF sporophore, on water agar, 7 days (790×).

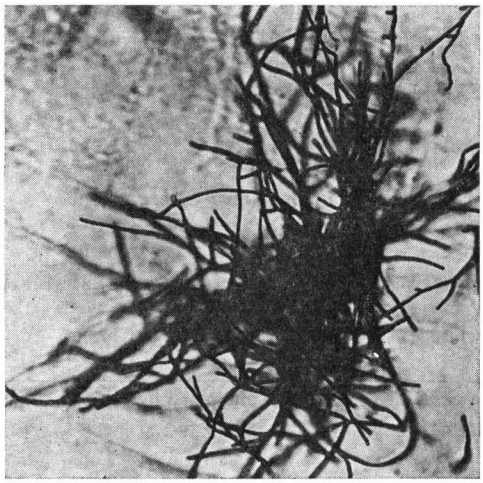


Plate 2. Photograph of aerial hyphae of isolate No. 4075, showing S sporophore, on sucrose-nitrate agar,7 days (937×).

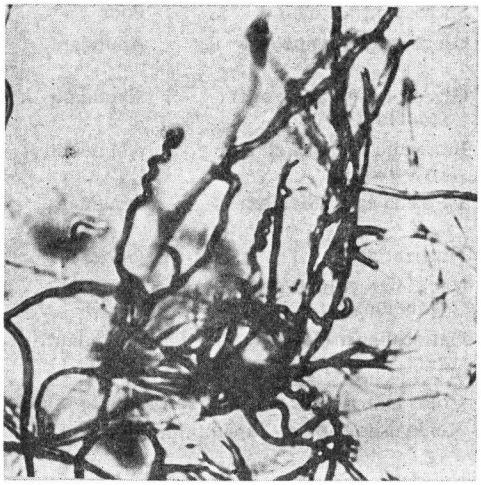


Plate 3. Electronmicrograph of spores of isolate No. 4075, showing smooth surface with minor irregularities, on water agar, 10 days.

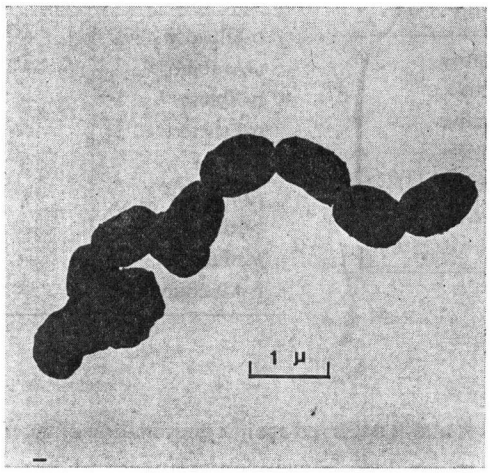
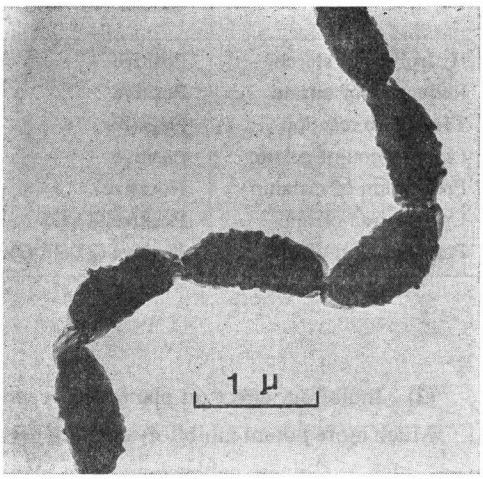


Plate 4. Electronmicrograph of spores of isolate No. 4075, showing warty surface, on yeast extract-malt extract agar, 14 days.



The antimicrobial spectra of the herbicidins against fungi are presented in Table 5. It is apparent from the results that both herbicidins A and B inhibit only *Trichophyton interdigitale* and *Pellicularia filamentosa*. There were also no activity at a concentration of 100 μg/ml of either herbicidin A or B against all of the bacteria tested, such as *Staphylococcus aureus* 209 P JC-1, *Micrococcus luteus* PCI 1001, *Bacillus subtilis* PCI 219, *Corynebacterium xerosis*, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ JC-2, *Proteus vulgaris* OX-19, *Klebsiella pneumoniae* PCI 602, *Pseudomonas aeruginosa* SANK 73860, *Aeromonas liquefaciens* and *Alcaligenes faecalis*. *Xanthomonas oryzae*, *X. campestris*, *X. citri* and *X. pruni* were also not susceptible to herbicidins at 100 μg/ml *in vitro*, but protection of the leaves of rice plant from bacterial leaf blight disease caused by *X. oryzae* H-5809 was demonstrated as shown in Table 6. Complete protection was performed by spraying 100 or 30 ppm either herbicidin A or B, respectively, and their effectiveness was still noted even at 3 ppm, the lowest concentration tested.

Table 1. Cultural characteristics of isolate No. 4075

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose-nitrate agar	Poor	Scant, white	White	None
Glucose-asparagine agar	Abundant	Good, yellowish gray	Light olive-gray to grayish olive	Light olive
Glycerol-asparagine agar (Medium 5)	Abundant	Poor, yellowish gray	Light yellow	None
Inorganic salts-starch agar (Medium 4)	Abundant	Good, white	Light yellowish brown	None
Tyrosine agar (Medium 7)	Abundant	Good, yellowish gray	Light yellow	None
Nutrient agar	Good	Poor, white	Light yellow	None
Yeast extract-malt extract agar (Medium 2)	Abundant	Good, yellowish gray	Yellowish brown	Yellowish brown
Oatmeal agar (Medium 3)	Abundant	Good, white	Light yellowish brown	None

Color names were assigned according to 'Guide to Color Standard', a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

Table 2. Physiological properties of isolate No. 4075 in comparison with those of *S. fluorescens* ISP 5203

	Isolate No. 4075	*S. fluorescens* ISP 5203
Hydrolysis of starch	Positive	Negative
Reduction of nitrate	Positive	Positive
Tyrosinase reaction	Negative	Negative
Liquefaction of gelatin	Positive	Positive
Production of melanin	Negative	Negative
Coagulation of milk	Positive (37°C)	Positive (25°C)
Peptonization of milk	Positive (25, 37°C)	Positive (25°C)

Table 3. Utilization of carbon source by isolate No. 4075.

Carbon source	Growth
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Fructose	+
Sucrose	+
i-Inositol	+
L-Rhamnose	+
Raffinose	−
D-Mannitol	+

(2) Inhibitory effect on plant-seed germination.

Much more potent inhibitory effect of herbicidins A and B occurred against germination of the plant seeds, such as Chinese cabbage and rice plant. The minimal inhibitory concentrations of both herbicidins A and B for germination of the seeds of Chinese cabbage were 0.78~1.56 and 1.56~3.12 μg/ml, respectively. Those for the seeds of rice plant were 6.25 and 12.5 μg/ml for A and B, respectively.

(3) Herbicidal activity.

Most characteristic feature of herbicidins A and B were demonstrated by their selective herbicidal activity when sprayed on the stems and leaves of the plants. As shown in Table 7, potent herbicidial activity was apparent especially against dicotyledonous plants. Among monocotyledonous plants, rice plant indicated strong resistance to herbicidins. Other species such as goose grass and other monocotyledonous plants were blighted by spraying herbicidins. Herbicidin A had a more selective effect between rice plant and other plants than herbicidin B.

(4) Toxicity test.

Herbicidins are markedly non-toxic. Mice tolerated intravenous dose of 800 mg/kg of herbicidin

Table 4. Comparison of cultural and physiological properties of isolate No. 4075 and *Streptomyces fluorescens* ISP 5203.

		Isolate No. 4075	*S. fluorescens* ISP 5203
1. Morphological characteristics	Aerial mycelium	Monopodial	Monopodial
	Sporophore	Rectiflexibilis to spira	Rectiflexibilis
	Surface of spore	Smooth to warty	Smooth
	Spores	Oval	Oval to cylindrical
2. Cultural characteristics (28°C)	Glycerol-asparagine agar	G: Abundant	G: Good
		AM: Yellowish gray	AM: White
		R: Pale yellow	R: Pale yellowish brown
		SP: None	SP: None
	Inorganic salts-starch agar	G: Abundant	G: Good
		AM: White	AM: White
		R: Pale yellowish brown	R: Pale yellowish brown
		SP: None	SP: None
	Yeast extract-malt extract agar	G: Abundant	G: Abundant
		AM: Yellowish gray	AM: Yellowish gray
		R: Yellowish brown	R: Yellowish brown
		SP: Yellowish brown	SP: None
	Oatmeal agar	G: Abundant	G: Good
		AM: White	AM: White
		R: Yellowish brown	R: Dull yellow
		SP: None	SP: Pale yellowish brown

G: Growth AM: Aerial mycelium R: Reverse SP: Soluble pigment

Table 5. Antimicrobial spectra of herbicidins A and B

Test organism	MIC (μg/ml)	
	A	B
Trichophyton interdigitale SANK 11968	50	100
Aspergillus oryzae IAM 2725	>100	>100
Penicillium chrysogenum NIH Q176	>100	>100
Saccharomyces cerevisiae ATCC 9763	>100	>100
Candida albicans YU 1200	>100	>100
Piricularia oryzae SANK 14758	>100	>100
Botrytis cinerea IAM 5126	>100	>100
Fusarium moniliforme IAM 5062	>100	>100
Pellicularia filamentosa SANK 22272	50	6.25
Alternaria kikuchiana F 42-5	>100	>100
Gloeosporium kaki KYU 438	>100	>100
Cochliobolus miyabeanus SANK 10458	>100	>100

Table 6. Effect of herbicidins on bacterial leaf blight disease caused by *Xanthomonas oryzae*.

	Concentration (ppm)	No. of leaves examined	Percent of diseased leaves	Toxicity to the host
Herbicidin A	300	315	0%	Negative
	100	322	0	Negative
	30	304	4	Negative
	10	311	18	Negative
	3	315	39	Negative
Herbicidin B	300	321	0	Slight discoloration of leaves
	100	309	0	Negative
	30	310	0	Negative
	10	320	5	Negative
	3	312	13	Negative
Untreated control	0	317	100	

Table 7. Herbicidal activity of herbicidins A and B.

		Herbicidin A (ppm)				Herbicidin B (ppm)			
		300	150	75	37.5	300	150	75	37.5
Mono-cotyledon	Rice	0	0	0	0	3	1	0	0
	Barnyard grass	3 *4	3 3	2 2	1 1	4 4	1 2	1 2	0 1
	Goose grass	*5	5	4	3	5	4	2	3
	Manna-grass	4 *3	4 3	3 2	2 0	5 3	4 3	2 2	2 1
	Green panicum	*4	2	1	1	3	3	2	1
Dicotyledon	Common purslane	*5	4	3	3	5	3	1	1
	Achyranthes	*5	5	5	4	5	5	4	4
	White goose-foot	*5	5	5	4	5	5	4	3
	Smartweed	*5	5	5	5	5	4	4	4
	Wild amaranth	*5	4	4	3	5	4	3	3
	Asiatic dayflower	*5	5	4	3	5	4	3	3
	Tomato	5	5	5	2	4	3	1	2
	Radish	5	5	4	3	5	4	2	3

The results, except for those with asterisks, were obtained from the experiment in which the plant seeds in unglazed flowerpots (9 cm in diameter) were covered with a soil of 10 mm depth, cultivated in a greenhouse for 2 weeks before application of herbicidin and examined the effect after 10 days. The data with asterisks were obtained from the same experiment as above, but on the plants seeds in 300-ml plastic pots were covered with a soil of 5-mm depth and herbicidal effect (0~5) was examined on the 15th day after application of the sample.

A or 200 mg/kg of B and intraperitoneal dose of 100 mg/kg of either A or B. No harmful effect on killifishes was observed after their incubation in a water bath containing 100 ppm of either A or B at room temperature for two days.

Glutarimide-group antibiotics, such as cycloheximide,[1] are known to have phytotoxic effects on various kinds of plants when sprayed on stems and leaves but a high concentration of the antibiotics is necessary to show its effectiveness. The non-selective killing effect between mono- and dicotyledonous plants as well as its toxicity prevents its use as weed killer. Inhibition of germination of plant seeds by anisomycin, deacetylanisomycin and toyocamycin are reported by MUNAKATA *et al.*[2,3] in which monocotyledonous plants including rice plant is more susceptible to the antibiotics than dicotyledonous plants. No contact herbicidal activity against plant is described for these antibiotics.

In contrast to these known antibiotics with herbicidal activity, herbicidins A and B were shown to have contact and selective activity, especially against dicotyledonous plants. This characteristic property of herbicidins may promise a possible usefulness of these antibiotics as weed killer.

References

1) NAKAMURA, K. & S. FUKUNISHI: Studies on the phytotoxicity. III. Classification of the phytotoxical symptom of barley and their relation to the chemical structure. Ann. Rep. Sankyo Res. Lab. 13: 245~249, 1961
2) MUNAKATA, K.: Herbicide. Japan Patent, 47–10076, March 25, 1972
3) ISHIDA, S.; O. YAMADA, F. FUTATSUYA, K. ITO, H. YAMAMOTO & K. MUNAKATA: New herbicides derived from an antibiotic, anisomycin. Proc. First Intersect. Congr. of IAMS. Vol. 3: 641~650, 1974

4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. System. Bacteriol. 16: 313~340, 1966
5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Internat. J. System. Bacteriol. 18: 318~319, 1968