PRODUCTION OF 9-β-D-ARABINOFURANOSYLADENINE BY A NEW SPECIES OF *STREPTOMYCES* AND ITS HERBICIDAL ACTIVITY

JUICHI AWAYA, KYOKO MATSUYAMA, YUZURU IWAI and SATOSHI ŌMURA*

The Kitasato Institute and Kitasato University, Minato-ku, Tokyo 108, Japan

HITOSHI ŌIWA and YASUHIRO HASEGAWA

Central Research Laboratory, Godoshusei Co., Ltd., 250 Nakahara, Kamihongo, Matsudo, Chiba, Japan

TAKASHI IKAI

Biological & Chemical Research Laboratory, Nissan Chemical Industries Ltd., 1470 Shiraoka, Minamisaitama Gun, Saitama, Japan

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In the course of our screening program for new biologically active metabolites from actinomycetes, we isolated a herbicidal metabolite from the culture filtrate of strain No. AM-3279, a soil isolate. The metabolite was identified as 9-β-D-arabinofuranosyladenine (ara-A) (**I**), which was at first prepared chemically from adenine by W. W. LEE *et al.*[1] and later isolated from the culture broth of *Streptomyces antibioticus*[2]. Strain AM-3279 was classified as a new species of *Streptomyces* and designated as *Streptomyces herbaceus* AWAYA and ŌMURA nov. sp.

This paper describes mainly taxonomic studies of the producing strain and herbicidal activity of ara-A.

Taxonomic Studies

Strain No. AM-3279 was isolated from a soil sample collected in Kitakomagun, Yamanashi Prefecture, Japan. Taxonomy of the strain was studied in accordance with the methods described by SHIRLING and GOTTLIEB[3], and WAKSMAN[4].

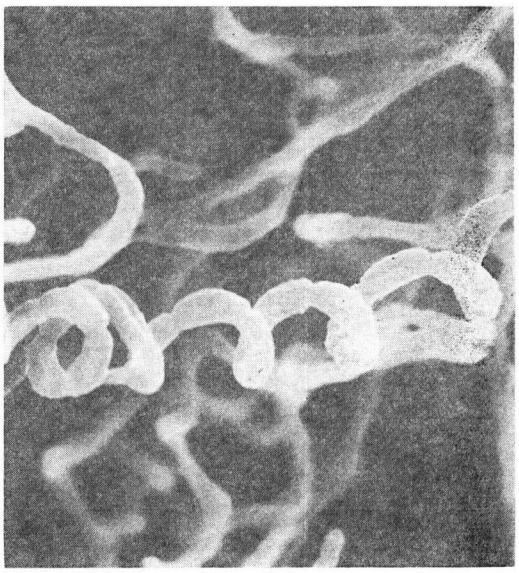
Morphological characteristics: The morphological characteristics of strain No. AM-3279 were observed on cultures incubated at 27°C for 14 days on oat meal agar, glycerol-asparagine agar and sucrose-nitrate agar. The aerial hyphae of the strain were simply branched. The sporophores were terminated by spore chains of moderately long, open coils (spiral) of three to five turns (Plate 1). On sucrose-nitrate agar, the coil numbers were more than five. Each spore chain with spirals had more than ten spores. Sclerotic granules and zoospores were not observed. Spores examined by electronmicroscopy, Plate 1, are oval in shape, $0.6 \times 1.0 \sim 1.1\ \mu$ in length and have a smooth surface.

Cultural characteristics: The cultural characteristics of strain No. AM-3279 shown in Table 1 were observed after two weeks of incubation at 27°C on various media. Color names and hue numbers are those of the Color Harmony Manual[5] published by Container Corporation of America.

Physiological characteristics: The physiological properties and the utilization of carbon sources are shown in Tables 2 and 3, respectively. Using the procedures described by BECKER *et al.*[6], the cell wall of strain No. AM-3279 was found to contain the LL-isomer of diaminopimelic acid and glycine.

The strain exhibits the following properties: The ends of aerial hyphae, spiral; spores, oval smooth surface; color of vegetative mycelia on various media, pale yellow, yellow or yellowish gray; color of aerial mycelium, pale green or green; melanin formation, none; analysis of cell

Plate 1. Electron micrograph of conidia of strain No. AM-3279 cultured on oatmeal agar for 14 days.



* To whom all correspondence should be addressed.

Table 1. Cultural characteristics of strain No. AM-3279.

Medium	Cultural characteristics
Sucrose-nitrate agar	Growth**(G): good, pale yellow (1ca) Reverse (R): parchment (ngs1cb) or chartreuse tint (ngs24½ba) Aerial Mycelium (AM): moderate, velvety; outer, white; inner, chartreuse tint (ngs24½ba) to parchment (ngs1cb) Soluble Pigment (SP): none
Glucose-nitrate agar	G: good, pale yellow (1ca) R: parchment (ngs1cb) or chartreuse tint (ngs24½ba) AM: moderate, velvety; outer, white; inner, chartreuse tint to parchment (ngs1cb) SP: none
Glycerol-asparagine agar (ISP)*	G: good, canary yellow (1ea) R: canary yellow (1ea); inner, light yellow (1½ea) AM: abundant, velvety, water drop, green tint (ngs24ba) to putty (ngs1dc) SP: mimosa yellow (1ia)
Glucose-asparagine agar	G: good, pearl (ngs2ba) R: yellow tint (ngs1ba); inner, canary yellow (1ea) AM: abundant, velvety, water drop, green tint (ngs24ba) to putty (ngs1dc); outer, white (a) SP: none
Glycerol-calcium malate agar	G: good, canary yellow (1ea) R: light lemon yellow (1ga) AM: moderate, velvety, white to pearl (ngs2ba) SP: light lemon yellow (1ga)
Inorganic salts-starch agar (ISP)*	G: good, yellow tint (ngs1ba) R: yellow tint (ngs1ba); inner, canary yellow (1ea) AM: abundant, velvety, green tint (ngs24ba) to white (a), sometimes partially putty (ngs1dc) SP: none
Tyrosine agar (ISP)*	G: good, canary yellow (1ea) R: canary yellow (1ea) and mimosa yellow (1ia) AM: abundant, velvety, white; inner, partially parchment (ngs1cb) SP: light lemon yellow (1ga)
Glucose-peptone agar	G: good, light ivory (2ca) R: cream (1½ca) AM: abundant, velvety, water drop, putty (ngs1dc), partially white (a) SP: none
Yeast extract-malt extract agar (ISP)*	G: good, light ivory (2ca) R: honey gold (2ic) AM: abundant, velvety, water drop, white (a); outer, yellow tint (ngs1ba) SP: none
Oatmeal agar (ISP)*	G: good, canary yellow (1ea) R: light lemon yellow (1ga) AM: abundant, velvety, water drop, parchment (ngs1cb) SP: none
Peptone-yeast extract iron agar (ISP)*	G: good, light wheat (2ea) R: canary yellow (1ea) AM: poor, velvety, water drop, white (a) SP: none
Nutrient agar	G: moderate, cream (1½ca) R: canary yellow (1ea) AM: moderate, velvety, white (a) SP: none

* Medium employed by International *Streptomyces* Project

** vegetative mycelium

Table 2. Physiological properties of strain No. AM-3279.

Temperature range for growth	20~37°C (opt. temp., 27~35°C)
Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	−
Hydrolysis of starch	+
Liquefaction of gelatin	−
Peptonization of milk	+
Coagulation of milk	−

Table 3. Utilization of carbon sources by strain No. AM-3279.

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

wall, positive for LL-diaminopimelic acid and glycine; grouping by key word[7], [GN; S; C-; SM].

From these data, it was concluded that strain No. AM-3279 belongs to the genus *Streptomyces*. A search of the classification keys for the genus *Streptomyces* in the standard references[7~11] showed no species to have the above-mentioned properties which are characteristic of strain No. AM-3279.

It was reasonable to conclude that strain No. AM-3279 is a new species of *Streptomyces* and designated as *Streptomyces herbaceus* AWAYA and ŌMURA nov. sp. The name *herbaceus* is derived from a Latin noun meaning "grass green" in English. The strain has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan and assigned as *Streptomyces herbaceus* with an accession number of FERM-P 4334.

Production, Isolation and Identification

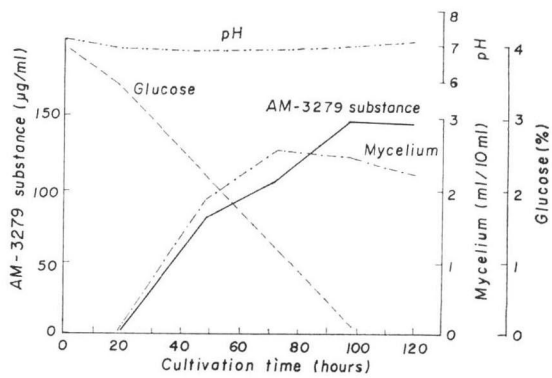
S. herbaceus was grown at 27°C for 7 days on a slant of glucose-asparagine agar. An aerial mass from the slant culture was placed into 10 ml of seed medium in a shake tube. A 72-hour shaking culture (2 ml) at 30°C was transferred into 100 ml of production medium in a 500-ml shake flask and incubated for 96 hours at 30°C on a rotary shaker operating at 240 rpm. The composition of seed and production medium was 4.0% glucose, 1.0% soybean meal, 0.25% yeast extract, 0.1% meat extract, 0.5% ammonium sulfate, 0.02% K_2HPO_4, 0.4% KCl and 0.3% $CaCO_3$. The pH of this medium was adjusted to 7.2 before autoclaving.

The antibiotic AM-3279 in the culture broth was estimated by the following chemical assay: Its Rf-value of the paper chromatogram (Toyo Roshi No. 50) in the solvent of *n*-butyl alcohol saturated with water is 0.28. Paper containing the fluorescent spot (Rf 0.28) under UV lamp was cut off and the antibiotic in this piece of paper was eluted with methanol. The amount of antibiotic AM-3279 was measured from the optical density of the methanol extract at 260 nm.

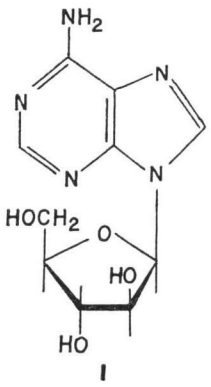
A typical time course of antibiotic AM-3279 production under the condition described above, is shown in Fig. 1. The antibiotic production started at about 25 hours and reached a maximum (144 μg/ml) at 96 hours after inoculation.

One liter of the resulting culture was centrifuged to obtain about 900 ml of culture supernatant. The supernatant was adjusted to pH 3.0 with 6 N hydrochloric acid and treated twice

Fig. 1. A typical time course of AM-3279 substance fermentation.



9-β-D-Arabinofuranosyladenine
(Ara-A)



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with successive 500 and 300 ml of ethyl acetate. The ethyl acetate layer was discarded. The aqueous layer was adjusted to pH 10.0 with 6 N sodium hydroxide and the antibiotic in aqueous solution was extracted twice with successive 500 and 300 ml of ethyl acetate. The combined extracts of the ethyl acetate layer were dried *in vacuo* to yield a crude powder (150 mg). The crude powder was chromatographed on silica gel (2 g) eluting with a mixture of chloroform-methanol (1: 1). The eluates were dried and yielded crude crystals. The crude crystals were recrystallized from hot methanol to give 30 mg of colorless crystals of antibiotic AM-3279 (melting point, 259~260°C).

The physico-chemical properties of the antibiotic indicated that it is most likely ara-A and its identity was confirmed by comparing it with an authentic sample in respect to melting point, behaviour on TLC, IR and mass spectrum. Consequently, antibiotic AM-3279 was identified as ara-A[1,2].

Herbicidal Activity

Plastic pots containing soil were planted with the seeds of various monocotyledonous and dicotyledonous plants and covered with soil to a depth of 5~10 mm and placed in a greenhouse. The following two treatments of ara-A (antibiotic AM-3279) were applied: The pre-emergence application (Table 4) was conducted before germination of these plants and the post-emergence application (Table 5) was conducted by spraying the plants two weeks after germination. The herbicidal effect was examined on the 15th or 20th day after the treatment with ara-A.

As shown in Tables 4 and 5, ara-A was more active to most of the plants in the pre-emergence application than in the post-emergence application. However, *Oryza sativa* (rice plant) had strong resistance against ara-A even in the pre-emergence application. Ara-A was found to have potent herbicidal activity against *Echinochloa crus-galli*, *Digitaria adscendens* and *Chenopodium ficifolium* in the pre-emergence application.

Table 4. Herbicidal activity of Ara-A (pre-emergence).

		Ara-A gram/are		
		100	50	25
Monocotyledon	*Oryza sativa*	0	0	0
	Echinochloa crus-galli	5	5	2
	Digitaria adscendens	5	4	2
Dicotyledon	*Chenopodium ficifolium*	5	4	3
	Postulaca oleracea	4	3	1
	Galinsoga ciliata	3	2	2
	Rorippa atrovirens	3	2	2

Herbicidal activity is evaluated by the following score of mortality (0~5): 0, no activity; 1, less than 20%; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, more than 90%.

Spraying condition: solvent, 0.1% polyethylene nonylphenyl-ether aqueous solution; concentration of ara-A, 5,000 ppm for 100 gram/are.

Table 5. Herbicidal activity of Ara-A (post-emergence).

		Ara-A gram/are		
		100	50	25
Monocotyledon	*Oryza sativa*	0	0	0
	Digitaria adscendens	3	2	2
	Cyperus microiria	2	1	0
Dicotyledon	*Chenopodium ficifolium*	4	3	3
	Postulaca oleracea	0	0	0
	Galinsoga ciliata	3	3	2

The evaluation of herbicidal activity and spraying of ara-A are carried out by the same manner as shown in Table 4.

Discussion

It has been known that ara-A originally synthesized from adenine by W. W. LEE *et al.*[1] and produced by *Streptomyces antibioticus*[2], has potent antiviral and antitumor activities and is a chemotherapeutic agent for herpes simplex viruses[12]. Its mode of action was reported to be the inhibition of DNA-dependent DNA polymerase[13].

In the course of our search for herbicidal antibiotics from soil cultures, we isolated ara-A from the culture filtrate of a new species of *Streptomyces* designated as *Streptomyces herbaceus* AWAYA and ŌMURA nov. sp. Among known nucleoside antibiotics, herbicidin[14,15] and toyocamycin[16] are known to have herbicidal activity. However, of interest is the new finding that ara-A has a potent and selective herbicidal activity against *Echinochloa crus-galli, Digitaria adscendens* and *Chenopodium ficifolium*. The antibiotic did not have herbicidal activity against *Oryza sativa*.

Summary

9-β-D-Arabinofuranosyladenine (ara-A) was produced by a new species of *Streptomyces* designated as *S. herbaceus*. Ara-A was found to have potent herbicidal activity against *Echinochloa crus-galli, Digitaria adscendens* and *Chenopodium ficifolium* by the treatment with ara-A before germination of these plants. However, *Oryza sativa* had strong resistance to ara-A.

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