7-HYDROXYGUANINE, A NOVEL ANTIMETABOLITE FROM A STRAIN OF *STREPTOMYCES PURPURASCENS*

I. TAXONOMY OF PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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Strain A-347, an actinomycete isolated from a soil sample, was found to produce a new antimetabolite, 7-hydroxyguanine. The aerial mycelium formed spiral spore chains with spiny spore surface. The chemical composition of strain A-347 indicated that it was an actinomycete of cell wall Type I. From its morphological, cultural, physiological characteristics and direct comparison with the type culture, this strain was classified as *Streptomyces purpurascens*.

7-Hydroxyguanine was purified from the broth filtrate by ion exchange chromatography and crystallized from hot 2 M NH_4OH. 7-Hydroxyguanine inhibited the growth of experimental tumors such as L1210 leukemia.

Antimetabolites constitute an interesting group of compounds used extensively in the chemotherapy of several types of neoplastic disease.

In our screening program for antimetabolites, 7-hydroxyguanine was isolated from a culture broth of actinomycete strain A-347. 7-Hydroxyguanine competed with purine bases and exhibited an antitumor effect on murine leukemia. In this paper, the taxonomy of the producing organism, fermentation, isolation, and biological properties of 7-hydroxyguanine are presented.

Taxonomic Studies of Strain A-347

An actinomycete strain, A-347, was isolated from a soil sample collected at Akashi City in Hyogo Prefecture, Japan. Morphological and physiological characteristics of strain A-347 were determined according to the procedures of SHIRLING and GOTTLIEB[1]; several other tests were also used. Observation of the culture was made after incubation at 28°C for 2 weeks. Color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo). The characteristics of strain A-347 were compared with those of the known species of actinomycetes described in the "ISP Report" by SHIRLING and GOTTLIEB[2], and "BERGEY's Manual of Determinative Bacteriology (8th edition)"[3]. Reference strains employed in the studies were received from the Institute for Fermentation, Osaka.

The cultural characteristics of strain A-347 are shown in Table 1. The color of the aerial mycelium was usually pale pink to pale orange. The physiological characteristics of strain A-347 are shown in Table 2. Cell wall analysis was performed by the method described by BECKER *et al.*[4,5]. The cell wall of strain A-347 contains LL-diaminopimelic acid; the whole cell hydrolysate contains glucose and

Table 1. Cultural characteristics of strain A-347.

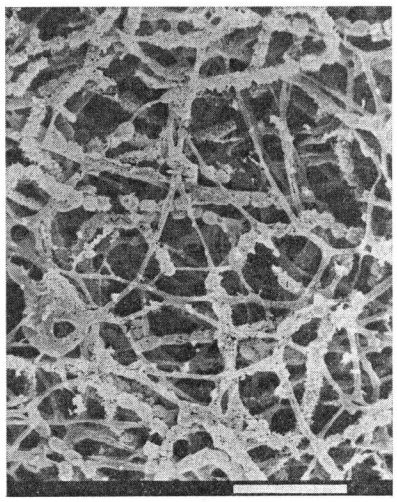
Medium	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract - malt extract agar (ISP 2)	Good: Pale orange to pale pink	Good: Light brown	None
Oatmeal agar (ISP 3)	Moderate: Pale orange to light brownish gray	Moderate: Pale yellowish orange	None
Inorganic salts - starch agar (ISP 4)	Poor: Pale orange	Moderate: Pale yellowish orange	None
Glycerol - asparagine agar (ISP 5)	Poor: Pale orange	Moderate: Pale yellowish orange	None
Tyrosine agar (ISP 7)	Poor: White	Moderate: Dark yellowish brown	Pale yellowish brown
Sucrose - nitrate agar	None	Poor: White	None
Glucose - asparagine agar	Moderate: Pale orange to pale pink	Good: Pale orange	None
Nutrient agar	None	Poor: White	None
Bennetts' agar	Moderate: Pale orange to pale pink	Moderate: Light olive gray	None

Table 2. Physiological properties of strain A-347.

Growth temperature: Medium 1	15~40°C
Gelatin liquefaction	−
Starch hydrolysis	+
Milk peptonization (26°C)	+
Milk coagulation (26°C)	−
Melanin formation: Medium 2	+
Medium 3	+
Nitrate reduction	+
NaCl tolerance: Medium 4	under 7%
Streptomycin tolerance: Medium 1	under 2 μg/ml
Carbon utilization	
D-Glucose	+
Glycerol	+
D-Xylose	+
L-Arabinose	+
Rhamnose	±
D-Fructose	+
Raffinose	+
D-Mannitol	+
Inositol	+
Sucrose	+

Medium 1: Waksman broth, Medium 2: peptone - yeast extract - iron agar (ISP 6), Medium 3: tyrosine agar (ISP 7), Medium 4: Waksman broth as basal medium.

Fig. 1. Scanning electron micrograph of aerial mycelium of *Streptomyces purpurascens* A-347.
Yeast extract - malt extract agar, 14 days culture.
Bar=5 μm.



ribose but lacks other diagnostic sugars. The above cell wall analysis indicates that strain A-347 belongs to the actinomycete of cell wall Type I. The spore and spore chain morphology of strain A-347 are shown in Figs. 1~3. The morphology of the spore chain is spiral, on glucose-asparagine agar and inorganic salts - starch agar and rectus flexibilis on yeast extract - malt extract agar. These results suggest that strain A-347 belongs to the genus *Streptomyces*.

According to the description of BERGEY's Manual, strain A-347 should be placed in the species group, *Spirales*, white, yellow or red series, chromogenic and spiny spore surface, which includes 11 species. Based on ISP species descriptions, strain A-347 resembles *Streptomyces hawaiiensis* ISP

5042, *S. janthinus* ISP 5206 and *S. purpurascens* ISP 5310. The results of direct comparison with type cultures indicated that strain A-347 most resembled *S. purpurascens* ISP 5310, reported as the producer of isorhodomycins[8], in morphology and cultural characteristics. In addition, the carbon source utilization profile of both strains was similar, the only difference was noted for antibiotic production. Therefore, strain A-347 was classified as *S. purpurascens*, and the culture has been deposited in the Fermentation Research Institute with the accession number FERM BP-541.

Fermentation

One loopful of strain A-347 growth was inoculated into a 500-ml Erlenmeyer flask containing

Fig. 2. Scanning electron micrograph of spore chain of *Streptomyces purpurascens* A-347.
Glucose - asparagine agar, 14 days culture.
Bar=5 μm.

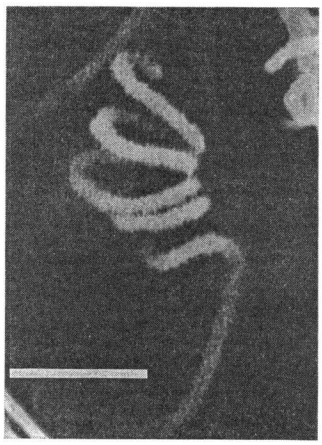


Fig. 3. Scanning electron micrograph of spore of *Streptomyces purpurascens* A-347.
Yeast extract - malt extract agar, 14 days culture.
Bar=0.5 μm.

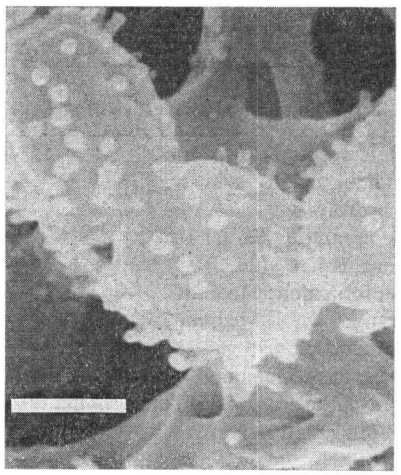
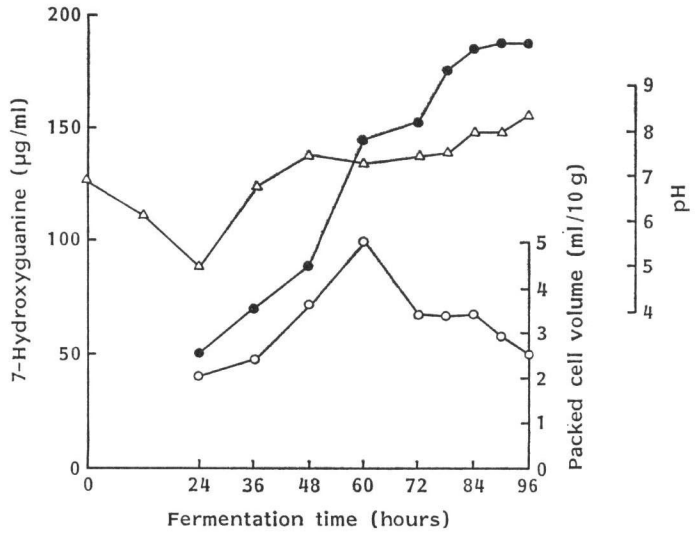


Fig. 4. Time course of 7-hydroxyguanine production in 2,000-liter fermentor.
● 7-Hydroxyguanine, ○ packed cell volume, △ pH.



100 ml of a seed medium composed of glucose 2.0%, sucrose 1.0%, soy bean meal 2.0%, L-asparagine 0.1% and $CaCO_3$ 0.05%. The pH of the medium was adjusted to 7.4 before sterilization. The flask was incubated on a rotary shaker at 28°C for 48 hours. A 900 ml-aliquot of the culture was inoculated into a 100-liter fermentor containing 60 liters of the same seed medium and the culture was incubated at 28°C for 48 hours with agitation of 350 rpm and aeration of 60 liters per minute. After inoculation of 15 liters of the seed culture into a 2,000-liter fermentor containing 1,000 liters of fermentation medium having the same composition as the seed medium, fermentation was carried out for 96 hours with agitation of 250 rpm and aeration of 320 liters per minute.

Mycelial growth was expressed as a packed cell volume (ml) after centrifugation of 10 g culture broth at 3,000 rpm for 15 minutes. The maximal potency of 188 μg/ml in supernatant was obtained after 96 hours of fermentation. The amount of 7-hydroxyguanine was determined by HPLC with a Nihon-bunko V system with Partisil 10 SCX column (4.6×250 mm) developed with a mobile phase of 10 mM $NH_4H_2PO_4$ buffer (pH 3.0) at a flow rate of 1.0 ml/minute. 7-Hydroxyguanine detected by UV absorption at 245 nm produced a single peak at 8.2 minutes of retention time. A time course of the fermentation in a 2,000-liter fermentor is shown in Fig. 4.

Isolation

Fermentation broth obtained as described above was adjusted to pH 3.5 and filtered through a bed of infusorial earth. The filtrate (840 liters, 176 μg/ml) was adjusted to pH 7.0. The antimetabolite was adsorbed on a column of Amberlyst 15 (H^+, 80 liters) and eluted with 0.5 M NH_4OH. The active eluate (202 liters, 520 μg/ml) was concentrated to 110 liters to remove NH_3 and an equal volume of deionized water was added. This diluted fraction (220 liters, pH 8.0) was applied to a column of Amberlite IRA 45 (OH^-, 20 liters) and eluted with 0.3 M NH_4OH. The active eluate (35 liters, 2,033 μg/ml) was concentrated to 17 liters and an equal volume of deionized water was added. This Amberlite purified fraction (34 liters, pH 8.0) was applied to a column of DEAE-Sephadex A-25 (HCO_3^-, 3 liters) and eluted with 0.3 M NH_4HCO_3. The active eluate (7 liters, 6,628 μg/ml) was concentrated to 1,560 ml at 65°C and placed in a refrigerator. The precipitate which formed overnight was collected, washed with acetone and dried *in vacuo* at room temperature.

The precipitate (35 g, 990 μg/ml) was dissolved in 700 ml of hot 2 M NH_4OH and placed in a refrigerator, yielding colorless needles of 7-hydroxyguanine (30.3 g, 20.5% yield).

Table 3. Antitumor activity of 7-hydroxyguanine against L1210 leukemia.

Dose (mg/kg/day)[a]	T/C (%)[b]
0.25	113
0.5	124
1.0	135
2.0	145
4.0	151
8.0	119

[a] Day 1~5 (ip).
[b] T/C (%) mean survival period of treated/mean survival period of control.

Biological Activity

Antimicrobial activity of 7-hydroxyguanine was investigated by a two-fold agar dilution method on Mueller-Hinton agar for bacteria and on SABOURAUD's dextrose agar for eumycetes. 7-Hydroxyguanine has no activity against *Staphylococcus aureus* 209P, *Bacillus subtilis* PCI 219, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* IFO 3445, *Micrococcus luteus* PCI 1001, *Candida albicans* 3147 and *Saccharomyces cerevisiae* at a concentration of 100 μg per ml.

7-Hydroxyguanine exhibited a prolongation

effect in the survival period of mice implanted with the mouse leukemia L1210 cells. Lymphoid leukemia L1210 was inoculated intraperitoneally into mice (female BDF_1 strain) with 10^5 cells per mouse. 7-Hydroxyguanine was administered to mice intraperitoneally 24 hours after tumor inoculation. The treatments were given once daily for 5 days.

The results are shown in Table 3.

The acute toxicity of 7-hydroxyguanine was determined in mice (male ICR strain) by single intraperitoneal administration, the LD_{50} being 40~80 mg/kg.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. Int. J. Syst. Bacteriol. 18: 69~189, 1968, 18: 279~392, 1968, 19: 391~512, 1969, 22: 259~374, 1972
3) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed., William & Wilkins Co., Baltimore, 1961
4) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography. Appl. Microbiol. 12: 421~423, 1964
5) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell wall preparations from strains of various form genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
6) LINDENBEIN, W.: Über einige chemish interessante Aktinomycetenstämme und ihre Klassifizierung. Arch. Mikrobiol. 17: 361~383, 1952