HISTIDINOMYCIN, A NEW ANTIFUNGAL ANTIBIOTIC

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A new antifungal antibiotic named histidinomycin was isolated from the broth filtrate of a streptomycete, tentatively designated as isolate H 878-MY 1. Histidinomycin was purified as the monohydrochloride and the molecular formula was proposed to be $C_{22}H_{30}N_8O_{11}\cdot HCl$. The antibiotic gave histidine by acid hydrolysis. Histidinomycin showed rather narrow antimicrobial spectrum for only few genera of phytophathogenic fungi.

In the course of our screening for new antifungal antibiotics, the broth filtrate of isolate H 878-MY 1 was shown to inhibit the growth of some imperfect fungi but did not inhibit that of yeasts. The purified antibiotic inhibited the growth of few restricted genera of phytophathogenic fungi such as *Glomerella*, *Colletotrichum* and *Elsinoë*. The antibiotic was proved to be new and named histidinomycin, because acid hydrolysis of the antibiotic gave histidine.

Taxonomic studies of isolate H 878-MY 1 and fermentation, extraction and purification, physicochemical and biological properties of histidinomycin are reported in this paper.

Taxonomic Studies

Isolate H 878-MY 1 was isolated from a soil sample collected in Nagasaki Prefecture, Japan.

Taxonomic studies on the isolate were conducted according to the procedures of the International Streptomyces Project (ISP)[1], WAKSMAN[2] and the BERGEY's Mannual of Determinative Bacteriology, 8th Ed.[3] and colors were described according to the color names and hue numbers of the Color Harmony Manual[4].

Isolate H 878-MY 1 produced aerial mycelia with spiral chains as seen in Fig. 1. The mature spore chains were generally long with 10 to 50 or more spores per chain and the spores were oval to cylindrical ($0.6\sim0.7\times1.0$ μm) with spiny surfaces as shown in Fig. 2.

Cultural characteristics of isolate H 878-MY 1 are listed in Table 1. Substrate mycelia grew moderate on many media except glycerol - asparagine agar (ISP-5) and color of substrate mycelia was

Fig. 1. Photomicrograph of isolate H 878-MY 1.

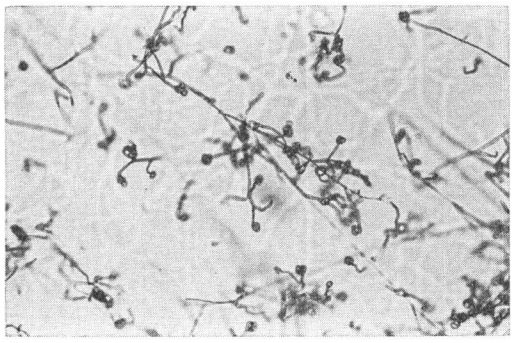


Fig. 2. Electron micrograph of spore chains of isolate H 878-MY 1.

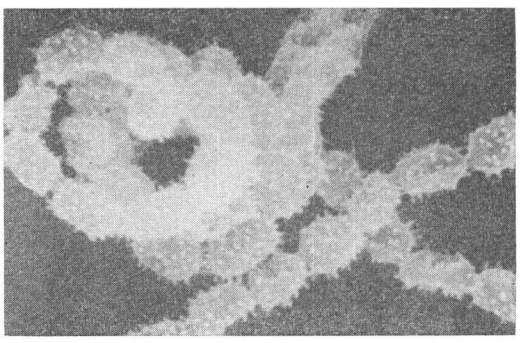


Table 1. Cultural characteristics of isolate H 878-MY 1.

	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Moderate White to shell pink (a ~ 5ba)	Moderate Butter scotch to golden brown (3ne ~ 3pg)	
Oatmeal agar (ISP-3)	Good (5cb)	Moderate Camel (3ie)	Pale brown
Inorganic salts - starch agar (ISP-4)	Good Dusty peach (5ec)	Moderate Cocoa brown (51g)	
Glycerol - asparagine agar (ISP-5)	None	Poor Colorless	
Glucose - asparagine agar	Good (5cb)	Moderate Camel to cinnamon (3ie ~ 3le)	Pale brown
Sucrose - nitrate agar	Moderate White to shell pink (a ~ 5ba)	Moderate Light spice brown to camel (41g ~ 3ie)	Pale brown

Table 2. Physiological characteristics of isolate H 878-MY 1.

Melanin production on		Carbon utilization	
Tyrosine agar (ISP-7)	Negative	D-Glucose	++
Peptone - yeast extract - iron agar (ISP-6)	Positive	D-Xylose	+
		L-Arabinose	++
Tryptone - yeast extract broth (ISP-1)	Negative	L-Rhamnose	++
		D-Fructose	++
H_2S production	Positive	D-Galactose	++
		Raffinose	++
Starch hydrolysis	Positive	Mannitol	++
Gelatin liquefaction	Positive	*meso*-Inositol	++
		Salicin	−
Skim milk		Sucrose	++
Coagulation	Negative	Cellulose	−
Peptonization	Positive		

++: Strongly positive utilization, +: positive utilization, −: negative utilization.

yellowish brown to brown. Aerial mycelia developed abundantly on oatmeal agar (ISP-3), inorganic salts - starch agar (ISP-4) and glucose - asparagine agar and the aerial mass color was pinkish white to pale reddish brown (Red color-series).

Physiological characteristics of isolate H 878-MY 1 are shown in Table 2. Melanoid pigments were formed in peptone - yeast extract - iron agar (ISP-6), but not in tyrosine agar (ISP-7) and Tryptone - yeast extract broth (ISP-1). Many carbon sources except salicin and cellulose were utilized by the isolate as seen in Table 2.

Cell wall analysis of isolate H 878-MY 1 according to the method of STANECK *et al.* revealed the presence of LL-diaminopimeric acid[5].

Isolate H 878-MY 1 was compared with descriptions of *Streptomyces*[2,3,6~9] in following criteria: melanoid pigments, produced; spore surface, spiny; aerial mass color, red color-series; spore chain morphology, section spirales. Consequently, isolate H 878-MY 1 was considered to be closely related to *Streptomyces thermotolerance*[10]. Essentially no difference was observed in carbon utilization between

isolate H 878-MY 1 and *S. thermotolerance*. Further, isolate H 878-MY 1 grew well on maltose-yeast extract or SABOURAUD's agar at 27, 37 and 40°C, but did not grow at all at 45°C and the optimum temperature was 37°C. On the other hand, isolate H 878-MY 1 produced the pale brown soluble pigment on several media as seen in Table 1. *S. thermotolerance* was reported to produce no or only trace of the yellow pigment. Nevertheless, the difference in productions of soluble pigments was considered to be not enough to differentiate both organisms.

Fermentation

Isolate H 878-MY 1 was cultured in 500-ml Sakaguchi flasks each containing 100 ml of a medium composed of 1% maltose and 0.2% yeast extract (pH was adjusted to 7.0~7.2 before sterilization) on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute) at 27°C for 48 hours. Each two ml of the culture was inoculated into 500 ml of Erlenmeyer flasks each containing 125 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast distributed by Tanabe Pharmaceutical Co. Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH was adjusted to 7.6 before sterilization). The fermentation was continued at 27°C for 90 hours on a rotary shaker (amplitude 7 cm, 175 strokes per minute). The antimicrobial activity was estimated by a paper disc-agar plate method using *Glomerella cingulata* as a test microbe on potato-sucrose agar.

Extraction and Purification

The active component was adsorbed on activated carbon from the broth filtrate and eluted with 80% aqueous acetone adjusting to pH 2.0. The eluted antibiotic was further purified by carbon column chromatography, CM-Sephadex column chromatography, precipitation as the reineckate and finally by preparative HPLC on reversed phase silica gel RP-8 (Lobar column, size B; distributed by E. Merck, Darmstadt) as shown in Chart 1. Analytical HPLC on Radial-PAK CN (distributed by Waters Associates, Mass.) eluted with MeOH - 0.01 M KH_2PO_4 (3: 17, adjusted to pH 3.0) was used to determine the purity of the antibiotic. The analytical HPLC was monitored with a UV detector at 210 nm and the retention time of the antibiotic was about 10 minutes at the flow rate of 2 ml per minute.

Physico-chemical Properties

Histidinomycin was isolated as the hydrochloride and recrystallized from MeOH to give white crystalline clusters, mp 181~183°C. $[\alpha]_D^{20}$ −46.4° (*c* 0.25, H_2O). The elementary analysis: Calcd for $C_{22}H_{30}N_8O_{11}\cdot HCl$: C 42.69, H 5.05, N 18.10 and Cl 5.73%. Found: C 42.12, H 5.21, N 18.04 and Cl 5.53%. The molecular formula, $C_{22}H_{30}N_8O_{11}$, was also supported by the mass spectrum (M^+ at *m/z* 582).

Histidinomycin hydrochloride showed UV spectra with absorption maxima at 215 nm ($E_{1cm}^{1\%}$ 175, sh) in 0.1 N HCl and at 219 nm ($E_{1cm}^{1\%}$ 180) in 0.1 N NaOH as seen in Fig. 3. IR spectrum of histidinomycin hydrochloride is shown in Fig. 4. The bands around 3300 cm^{-1} are due to –OH and –NH groups and the existence of amide groups are suggested by the bands around 1640 and 1540 cm^{-1}.

Table 3. Chromatographic behaviors of histidinomycin (hydrochloride) on silica gel thin-layer plates.

Solvent system	Rf value
1-BuOH - AcOH - H_2O (2: 1: 2)	0.22
1-BuOH - AcOH - H_2O (3: 2: 2)	0.14
1-BuOH - AcOH - H_2O (2: 1: 1)	0.08
1-BuOH - pyridine - H_2O (6: 4: 3)	0.09
1-PrOH - pyridine - AcOH - H_2O (15: 10: 3: 12)	0.54
1-PrOH - AcOH - H_2O (25: 2: 20)	0.16
1-PrOH - MeOH - H_2O (1: 1: 2)	0.14
1-PrOH - acetone - H_2O (1: 1: 1)	0.08

TLC plates Kieselgel 60 F_{254} (distributed by E. Merck, Darmstadt) were used.

Chart 1. Extraction and purification of histidinomycin.

Broth filtrate (12,500 ml, pH 7.6)
↓ stirred with activated carbon (87.5 g) for 1 hour and filtered
Activated carbon | Filtrate (12,350 ml, total activity 6.3%)
↓ washed with H_2O (500 ml) and eluted twice with 80% aq acetone adjusting to pH 2 by addition of dil HCl
First eluate (1,340 ml, total activity 88%)
+
Second eluate (690 ml, total activity 6.5%)
↓ pH was adjusted to 6 and concentrated *in vacuo*
First concentrate (420 ml, total activity 84%)
+
Second concentrate (215 ml, total activity 6%)
↓ pH was adjusted to 7
Carbon column (35 g, 56×2.5 cm, diameter)
↓ eluted with a linear gradient concentration of H_2O (1,000 ml)←80% aq acetone - 1 N HCl (9: 1) (1,000 ml) and collected in 19.5 ml fractions each
Fractions No. 41~63 (448 ml, total activity 81%)
↓ concentrated *in vacuo* after pH was adjusted to 5~6
Concentrate (165 ml, total activity 81%)
↓ pH was adjusted to 6.8
CM-Sephadex column (16×2.4 cm, diameter)
↓ washed with H_2O (100 ml), eluted with a linear gradient concentration of H_2O (500 ml)← 0.5 M NaCl (500 ml) and the eluate was collected in 20 ml fractions each
Fractions No. 8~16 (180 ml, total activity 78%)
↓ pH was adjusted to 7
Carbon column (3 g, 16.5×1.2 cm, diameter)
↓ eluted with a linear gradient concentration of H_2O (300 ml)←acetone - 0.1 N HCl (8: 2) (300 ml) and collected in 10 ml fractions each
Fractions No. 23~30 (80 ml, total activity 74%)
↓ pH was adjusted to 6 and concentrated to 10 ml *in vacuo*
Concentrate (10 ml, total activity 74%)
↓ added saturated aq ammonium reineckate
Precipitate | Filtrate (17 ml, total activity 2%)
↓ dissolved in H_2O (20 ml) at 60°C and an aq solution of pyridine hydrochloride was added
Filtrate (20 ml, total activity 68%) | Precipitate (pyridine reineckate)
↓ pH was adjusted to 7
C-M Sephadex column (16×2.4 cm, diameter)
↓ treated with the same procedures as described before
Fractions No. 8~16 (80 ml, total activity 63%)
↓ pH was adjusted to 7
Carbon column (3 g, 16.5×1.2 cm, diameter)
↓ eluted with the same procedures as described before
Fractions No. 22~25 (40 ml, pH 4.6~4.8, total activity 48.6%)
+
Fraction No. 26 (10 ml, pH 4.0, total activity 6.6%)
↓ concentrated *in vacuo* and lyophilized
Fractions No. 22~25 (67.0 mg, total activity 47.2%)
+
Fraction No. 26 (28.7 mg, total activity 6.3%)

Chart. 1. (continued)

Fractions No. 22~25 (38.1 mg, total activity 100%)
| dissolved in H_2O (1.9 ml)
Lobar column RP-8 (size B, 310×25 mm, diameter)
| eluted with aq 30% MeOH at the flow rate of 2 ml per minute and the eluate was collected in 15 ml fractions each
Fractions No. 5, 6
+
Fractions No. 7, 8
+
Fractions No. 9~26
| concentrated *in vacuo* and lyophilized
Fractions No. 5, 6 (11.2 mg, total activity 13.5%)
+
Fractions No. 7, 8 (7.7 mg, total activity 10.6%)
+
Fractions No. 9~26 (20.1 mg, total activity 70.0%)
Fractions No. 9~26 (11.4 mg)
| dissolved in MeOH (4 ml), kept in a refrigerator for 48 hours and filtered
Crystalline clusters of histidinomycin hydrochloride (7.8 mg)

The hydrochloride was soluble in H_2O, sparingly soluble in MeOH and insoluble in ethyl acetate, acetone, chloroform and ether. Chromatographic behaviors of the antibiotic on silica gel thin-layer plates are listed in Table 3.

Histidinomycin gave positive ninhydrin, naphthoresorcinol - phosphate (light brown), Cl_2 - I_2 - starch, Elson-Morgan (yellow) and Pauly reactions, but negative Tollens reaction. Histidinomycin was stable in a solution at pH 4~6 when kept at 100°C for 5 minutes. While, approximately 60% or 30% of the original antimicrobial activity was lost in a solution at pH 9 or pH 2, respectively, under the same condition. The antibiotic was hydrolyzed to yield ninhydrin positive constituents including amino acids

Fig. 3. Ultraviolet absorption spectra of histidinomycin (hydrochloride).

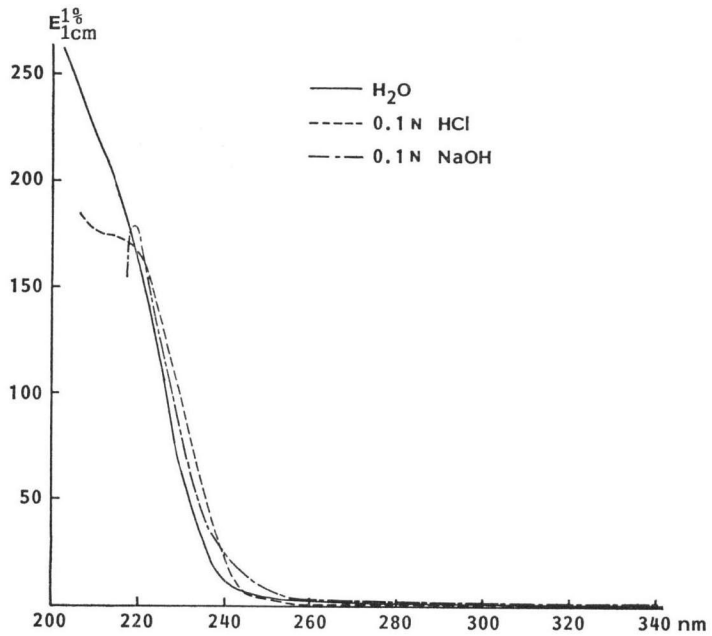
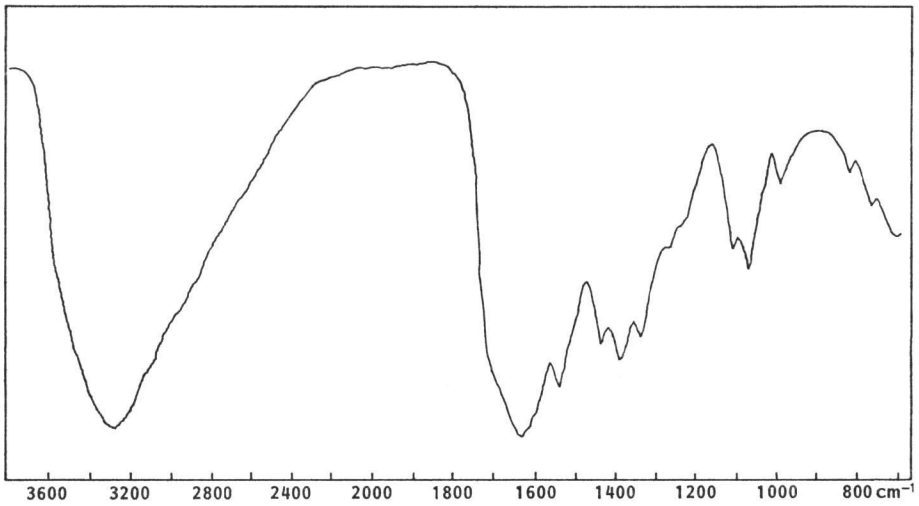


Fig. 4. Infrared absorption spectrum of histidinomycin (hydrochloride) in KBr.



by the treatment with constant-boiling HCl at 110°C for 17 hours in a sealed tube. The hydrolyzed mixture gave six spots on a silica gel TLC plate (Kieselgel 60 F_{254}, distributed by E. Merck, Darmstadt) developed with 1-BuOH - AcOH - H_2O (2: 1: 1). The Rf values are as follows; 0.43 (pale purple, very weak), 0.39 (pale reddish orange, weak), 0.35 (pale purple), 0.29 (purple), 0.26 (yellow orange) and 0.18 (brown purple). The brown purple spot at Rf 0.18 was proved to be histidine by the TLC. Thus, the antibiotic was named histidinomycin.

Biological Properties

The antimicrobial activity of histidinomycin was tested by the cylinder agar plate method against *G. cingulata* on potato - sucrose agar. The antibiotic showed turbid inhibition zones of 15.5, 20.0 and 24.5

Table 4. Antimicrobial spectrum of histidinomycin.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)	
			36 hours	66 hours
Staphylococcus aureus FDA 209P	>100	*Alternaria kikuchiana*	100	>100
Micrococcus luteus PCI 1001	>100	*Glomerella cingulata*	0.025	0.78
Micrococcus flavus FDA 16	>100	*G. cingulata* No. 3	>100	>100
Bacillus subtilis PCI 219	>100	*Colletotrichum lagenarium*	0.1	0.39
Mycobacterium smegmatis ATCC 607	>100	*C. gloeosporioides*	0.0125	0.39
Corynebacterium bovis 1810	>100	*C. lindemuthianum*	0.0016	0.0063
Escherichia coli NIHJ	>100	*Gloeosporium laeticolor*	1.56	>100
Serratia marcescens	>100	*Elsinoë fawcettii*	0.1	0.39
Salmonella typhi T-63	>100	*Trichophyton mentagrophytes*	>100	>100
Shigella sonnei 191–66	>100	*T. asteroides*	>100	>100
Klebsiella pneumoniae PCI 602	>100	*Aspergillus niger* F-16	>100	>100
Pseudomonas aeruginosa Ishii 14	>100	*Pellicularia filamentosa*	—	0.1
Xanthomonas oryzae N 5824	>100	*Helminthosporium oryzae*	>100	>100
Candida albicans 3147	>100	*Cryptococcus neoformans*	>100	>100
Saccharomyces cerevisiae	>100	*Pyricularia oryzae*	>100	>100

MIC were determined by agar dilution method. Bacteria and yeasts were tested on glucose nutrient agar. Fungi were tested on potato - sucrose agar.

cm (diameters) at concentrations of 0.0019, 0.0078 and 0.031 μg/ml, respectively.

The antimicrobial spectrum of histidinomycin was determined by agar dilution method as shown in Table 4. Histidinomycin inhibited the growth of only few restricted genera of phytophathogenic fungi such as *Glomerella*, *Colletotrichum* and *Elsinoë*, but was inactive against bacteria and yeasts so far tested.

Discussion

Histidinomycin is a kind of basic water soluble peptide antibiotics and contains five ninhydrin positive amino acids and amino sugars besides histidine. The antibiotic is unique among low molecular peptide antibiotics because of containing histidine and showing the very narrow antimicrobial spectrum against a few genera of phytophathogenic fungi. Histidomycins A and B produced by *Nocardia histidans* are also low molecular antibiotics, but histidomycins are active against Gram-positive and negative bacteria[11,12]. Up to date, further, no antibiotic having the molecular formula of $C_{22}H_{30}N_8O_{11}$ has been reported in literatures. Thus, histidinomycin is considered to be a new antibiotic.

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