Pyrindicin, a New Alkaloid from a *Streptomyces* Strain

Taxonomy, Fermentation, Isolation and Biological Activity†

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In the course of our examination for the alkaloid productivities of *Streptomyces* strains, *Streptomyces* sp. NA-15 was found to produce a new alkaloid, pyrindicin, in the culture medium. The strain NA-15 was found to be a variant of *Streptomyces griseoflavus* and was designated as *S. griseoflavus* var. *pyrindicus* nov. var.

After the culture conditions for pyrindicin production were studied, pyrindicin was obtained as its hydrochloride (mp 145°C, decomp.) from the cultured broth. The compound was shown to possess weak antimicrobial and several pharmacological activities. The LD₅₀ of the hydrochloride (ip, in mice) was 87 mg/kg.

It is well known that a large number of alkaloids are produced by various plants. But in microorganisms, no alkaloids has been found for years except for ergot alkaloids from ergot fungi and a few others.^{1~4)} Meanwhile, it was reported that different alkaloids are produced by molds and bacteria.^{5~10)} Kaneko and Terashima^{11~14)} found also that *Streptomyces nigrifaciens* var. FFD-101 produced a new alkaloid, nigrifactin, in the culture medium.

In the course of the examination for the alkaloid productivities of *Streptomyces* strains, *Streptomyces* sp. NA-15 was found to produce a new alkaloid, which was named pyrindicin from the fact that the structure was a 1-pyrindine derivative.¹⁵⁾ This paper deals with the taxonomical studies of *Streptomyces* sp. NA-15 as well as with the isolation and biological activities of pyrindicin. In addition, the culture conditions essential for pyrindicin production are described. The chemical structure of pyrindicin was reported in a separate paper by M. Onda *et al.*¹⁵⁾

MATERIALS AND METHODS

Microorganisms. *Streptomyces* strains employ were obtained from our laboratory stock or isolated from soil samples. Among them, *Streptomyces* sp. NA-15 was studied extensively.

Media and culture conditions. For the screening test, an organism grown on an agar slant (glucose 1.0%, asparagine 0.05%, K₂HPO₄ 0.05%, agar 1.5%) was transferred into a 50 ml-test tube containing 15 ml of a liquid medium shown in Table I, and incubated

TABLE I. COMPOSITION OF THE MEDIUM FOR THE SCREENING TEST

After adjusting to pH 7.0, the medium was autoclaved for 10 min at 121°C.

Component	Concn. (%)
Glucose	2.0
Peptone	0.5
KH ₂ PO ₄	0.1
MgSO ₄ ·7H ₂ O	0.05
NaCl	0.5

on a reciprocal shaking machine for 5 days at 27°C.

For the production of pyrindicin, two milliliters of the seed culture of *Streptomyces* sp. NA-15 grown on a liquid medium shown in Table II for 2 days at 27°C was transferred into a 500 ml-Sakaguchi flask containing 125 ml of the same medium, and incubated

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TABLE II. COMPOSITION OF THE MEDIUM FOR PYRINDICIN PRODUCTION

After adjusting to pH 7.0, the medium was autoclaved for 10 min at 121°C.

Component	Concn. (%)
Glucose	2.0
Peptone	0.5
Meat extract	0.5
Dry yeast	0.3
CaCO ₃	0.3
NaCl	0.5

on a reciprocal shaking machine for 4~5 days at 27°C.

Primary screening test. A culture filtrate was divided into two parts. One part was used for the primary test, and the other for the secondary one. After adjusting the pH to 4.0 with 0.1 N hydrochloric acid, a part of the culture filtrate was tested with Dragendorff's reagent according to Munier and Macheboeuf.¹⁶⁾ Formation of precipitates showed the positive result.

Secondary screening test. If the reaction in the primary screening test was positive, a secondary screening test was conducted on the reserved culture filtrate. Five milliliters of the filtrate was made alkaline with ammonia water and extracted with 1 volume of ethyl acetate. The ethyl acetate layer was extracted with 1 ml of 0.1 N hydrochloric acid and tested with the same reagent.

Taxonomic studies. *Streptomyces* sp. NA-15 was cultivated on various media described by ISP (International *Streptomyces* Project)¹⁷⁾ and Waksman¹⁸⁾ at 27°C or 37°C, and at the period of 7, 14 and 21 days, the changes of growth, aerial mycelium and soluble pigment were observed. Morphological properties of the strain were studied on glycerol-asparagine agar under a microscope and a scanning electron microscope (JSM-2, JEDL Co., Ltd.). Utilization of carbon sources was tested by growth on Pridham and Gottlieb's medium¹⁷⁾ containing 1% of various carbon sources.

Assay of alkaloid accumulated in the culture medium. After the culture filtrate (50 ml) was made alkaline with ammonia water, butyl acetate (25 ml) was added to the filtrate and then mixed vigorously. The butyl acetate layer was extracted with 0.1 N hydrochloric acid (5 ml), and serial two-fold dilutions of the extract were made. The diluted solutions were tested with Dragendorff's reagent. Titer (units) of alkaloid was indicated by maximum dilution number of the positive solutions.

Assay of mycelial growth. After 10 ml of cultured broth was centrifuged for 10 min at 3000 rpm, the wet volume of mycelia precipitated was measured.

Assay of residual glucose. Glucose remained in the cultured medium was determined by *o*-toluidine method.¹⁹⁾

Thin-layer chromatography. Silica gel G (Merck) plates were used in this work. A solvent system, *n*-butanol-acetic acid-water (4:1:1), was employed extensively. The detection of alkaloid was done by spraying Dragendorff's reagent according to Munier and Macheboeuf.¹⁶⁾

RESULTS

I. Examination for the alkaloid productivities of *Streptomyces* strains

Streptomyces strains (815 strains) from our laboratory stock and from soil samples, the culture filtrates of which had no or weak antimicrobial activities, were tested for alkaloid production in the culture medium. In the primary screening test, the culture filtrates of 164 strains among the tested *Streptomyces* strains were positive against Dragendorff's reagent. In the secondary screening test, 17 strains were selected as alkaloidal substance-producing strains. Among 17 strains selected, *Streptomyces* sp. NA-15 produced a largest amount of an alkaloidal substance. The substance was found to be a new compound, and was named pyrindicin. In the following sections, taxonomical studies of the strain, fermentation, isolation, characterization and biological activities of pyrindicin are described.

II. Characteristics of the pyrindicin-producing strain

The cultural and physiological characteristics of the strain NA-15 are listed in Tables III and IV. The utilization of carbon sources by the strain is shown in Table V. The characteristics of this strain can be summarized as follows: it forms no whorls, but extends aerial hyphae forming open or compact spirals; surface of spore is spiny; growth is colorless to creamy on various media; aerial mass color is white to light gray on various media; soluble pigment is pale yellow or yellowish brown on various

TABLE III. CULTURAL CHARACTERISTICS OF *Streptomyces* sp. NA-15.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	good, colorless to grayish white	pale yellow	abundant, white to whitish gray	pale yellow
Glucose-nitrate agar	good, colorless to grayish white	light yellow	abundant, white to whitish gray	grayish yellow
Glycerol-asparagine agar	good, dull yellowish orange	light brown	scant, white to gray	grayish brown
Inorganic salt-starch agar	good, colorless	light brown	abundant, whitish gray	brown
Tyrosine agar	good, colorless	yellowish gray	brownish gray	grayish yellow
Nutrient agar	creamy	light yellow	none	pale yellow
Glucose-peptone agar	moderate, creamy	creamy	scant, white	pale yellow
Yeast extract-malt extract agar	good, colorless	pale yellowish brown	abundant, brownish gray, with drop	pale yellowish brown
Oat meal agar	good, colorless	yellowish gray	white to gray	grayish and yellowish brown
Peptone-yeast extract iron agar	good, colorless to creamy	pale yellow	scant, grayish white	pale yellowish brown
Tryptone-yeast extract broth	surface growth, colorless to creamy		white	none
Milk	ring, creamy		scant, white to gray	yellowish orange
Gelatine	creamy		scant, white	none
Cellulose	thin		gray	none

TABLE IV. PHYSIOLOGICAL PROPERTIES OF *Streptomyces* sp. NA-15

Melanin formation	negative
Tyrosinase reaction	negative
Hydrolysis of starch	positive
Liquefaction of gelatin	positive
Peptonization of milk	positive
Coagulation of milk	negative
Cellulolytic activity	positive
Temperature range for growth	15~45°C
Optimum temperature for growth	25~35°C
pH range for growth	4~9
Optimum pH range for growth	6~8

TABLE V. UTILIZATION OF CARBON SOURCES BY *Streptomyces* sp. NA-15

Response	Carbon source
Positive	glucose, fructose, xylose, arabinose, maltose, mannose, rhamnose, inositol, mannitol, starch
Negative	sucrose, raffinose

media; it produces no melanoid pigment (non-chromogenic type). Among known *Streptomyces* species described in "Bergey's Manual of Determinative Bacteriology," 7th ed.²⁰⁾ and "The Actinomycetes," Vol. II.¹⁸⁾ *Streptomyces griseoflavus* (Krainsky) Waksman and Henrichi was closely related to the strain NA-15. In comparison of the strain NA-15 with the type culture, *Streptomyces griseoflavus* ISP 5456 (KA 1064), the properties of the former were almost in agreement with those of the latter. However, as shown in Table VI, such characteristics of the former as the color of vegetative mycelium and the formation of soluble pigment on some media were slightly different from those of the latter. And also the type culture ISP 5456 did not produce this new alkaloid, pyrindicin. Therefore, the strain NA-15 should be assigned to a variant of *Streptomyces griseoflavus* (Krainsky) Waksman and Henrichi, and was designated as *Streptomyces griseoflavus* var. *pyrindicus* Awaya

TABLE VI. COMPARISON OF *Streptomyces* sp. NA-15 AND *S. griseoflavus* ISP 5456

		<i>Streptomyces</i> sp. NA-15	<i>S. griseoflavus</i> ISP 5456
Morphological properties			
Spore chain		spiral	spiral
Spore surface		spiny	spiny
Cultural properties			
Sucrose-nitrate agar	{ G AM SP	colorless or grayish white white to whitish gray pale yellow	colorless whitish gray none
Yeast extract-malt extract agar	{ G AM SP	colorless brownish gray pale yellowish brown	creamy to pale yellowish brown brownish gray yellowish brown
Oat meal agar	{ G AM SP	colorless or creamy brownish gray yellowish brown	colorless brownish gray none
Inorganic salt-starch agar	{ G AM SP	colorless whitish gray brown	yellow to pale yellowish brown yellowish brown none
Glycerol-asparagine agar	{ G AM SP	dull yellowish orange white to gray grayish brown	creamy to pale yellowish brown pale yellowish gray pale yellow
Glucose-peptone agar	{ G AM SP	creamy white pale yellow	yellowish yellowish white yellowish brown
Physiological properties			
Reduction of nitrate		positive	positive
Production of pyrindicin		positive	negative

G: growth, AM: aerial mycelium, SP: soluble pigment.

and Hata on the basis of the fact that it produces pyrindicin.

III. Culture conditions for pyrindicin production by *Streptomyces* sp. NA-15

Since the amount of pyrindicin accumulated by *Streptomyces* sp. NA-15 was small, the culture conditions were investigated for the production of the alkaloid. The alkaloid accumulated in the culture medium was assayed as described above, and checked upon by thin-layer chromatography.

Carbon sources. The relations between carbon sources and pyrindicin production were examined. When the medium containing peptone 0.5%, meat extract 0.5%, CaCO_3 0.3% and NaCl 0.5% was used as the basal medium, glucose (2%) gave the best result for the production as shown in Table VII.

Nitrogen sources. The relations between natural nitrogen sources and pyrindicin production were examined using the medium

TABLE VII. RELATIONS BETWEEN CARBON SOURCES AND PYRINDICIN PRODUCTION BY *Streptomyces griseoflavus* var. *pyrindicus* nov. var.

Basal medium: peptone 0.5%, meat extract 0.5%, CaCO_3 0.3%, NaCl 0.5%, pH 7.0.

Carbon source (%)	Titer of pyrindicin produced (units/ml)
Glucose { 2 10	256 128
Mannitol { 2 10	128 64
Glycerol { 2 10	128 128
Starch 2	128

containing glucose 2.0%, CaCO_3 0.3% and NaCl 0.5% as the basal medium. As shown in Table VIII, the amount of the alkaloid produced was larger when soybean meal was used, and smaller when peptone or meat extract alone was used. However, when soybean meal was used, a by-product was produced. Therefore, the medium containing peptone 0.5%, meat extract 0.5% and dry yeast 0.3%

as nitrogen sources was used for the following experiments.

Antiform concentration. An antiform was not necessary for the fermentation in Sakaguchi flask, but it was necessary in a jar or tank fermentor. Effects of an antifoam, Adekanol LG-109 (obtained commercially from Asahi Electro-Chemical Co., Ltd.) on pyrindicin production were examined. As shown in Table IX, the growth, the pellet formation and the alkaloid production were inhibited by Adekanol at the concentration over 0.025%. But, 0.008% of adekanol still exhibited a sufficient antifoam activity.

Time course of pyrindicin production. Under the optimal conditions according to the

above data, the fermentation was carried out using a 30-liter jar fermentor. The time course of a typical fermentation is shown in Fig. 1. The pyrindicin production was observed from about 2 days after the inoculation and the maximum concentration was reached at about 3 days. After glucose disappeared at 3 days, the pyrindicin concentration decreased.

IV. Isolation and chracterization of pyrindicin accumulated in the culture medium

Cultured broth (70 liters) of *Streptomyces* sp. NA-15 obtained by incubation in a jar fermentor was used as a starting material for the isolation of pyrindicin; the titer of the broth was 256 units/ml. After the broth filtrate was

TABLE VIII. RELATIONS BETWEEN NITROGEN SOURCES AND PYRINDICIN PRODUCTION BY *Streptomyces griseoflavus* var. *pyrindicus* nov. var.

Basal medium: glucose 2.0%, CaCO₃ 0.3%, NaCl 0.5%, pH 7.0

No.	Nitrogen sources ^{a)} (%)					Growth	Titer of pyrindicin (units/ml)
	P	SBM	ME	DY	YE		
1	0.5 ²	—	0.5	0.3	—	++	128
2	2.0	—	—	—	—	+	64
3	1.0	—	—	—	—	+	64
4	0.5	—	—	—	—	+	64
5	—	2.0	—	—	—	+	192
6	—	1.0	—	—	—	+	192
7	—	—	2.0	—	—	++	64
8	—	—	1.0	—	—	+	32
9	—	—	0.5	—	—	±	24
10	0.5	—	0.5	—	—	++	32
11	—	0.5	0.5	—	—	++	256
12	0.5	0.5	—	—	0.5	+	64

^{a)} Abbreviations used: P, peptone; SBM, soybean meal; ME, meat extract; DY, dry yeast; YE, yeast extract

TABLE IX. EFFECTS OF THE CONCENTRATION OF AN ANTIFOAM, ADEKANOL LG-109, ON PYRINDICIN PRODUCTION

Basal medium: shown in Table II

Concn. of Adekanol (%)	Antifoam activity	Growth	Pellet formation	Titer of alkaloid (units/ml)
None	negative	good	abundant	128
0.008	positive	good	abundant	128
0.016	positive	good	abundant	128
0.025	positive	poor	no	64
0.04	positive	poor	no	64
0.08	positive	poor	no	64

FIG. 1. Time Course of the Pyrindicin Production by *Streptomyces griseoflavus* var. *pyrindicus* nov. var.

Cultivation was performed using a 30-liter jar fermentor containing 20 liters of the medium as shown in Table II. Adekanol (0.015%) was added before inoculation. Culture conditions were as follows: agitation 300 rpm, temp. 27°C, and aeration 10 liters/min.

●—●, Alkaloid accumulation; ○—○, mycelial growth; ○—○, residual glucose; ×—×, pH.

made alkaline with ammonia water, the alkaloid was extracted with one-fifth volume of butyl acetate and then transferred into one-fifth volume of 0.1 N hydrochloric acid. The aqueous layer was adjusted alkaline and a one-half volume of ethyl acetate was added. The solvent layer was dehydrated with anhydrous sulfate and concentrated under reduced pressure to one-fifth volume. As the free alkaloid was very labile and easily oxidized to a brown syrup, it could not be obtained in a crystalline state. In order to get the alkaloid in a pure state, it was converted into its crystalline salts of picric acid. Picric acid-saturated ethyl ether was added to the above-mentioned concentrate until no more crystallization or precipitation occurred. Yellow needles (pyrindicin picrate) were obtained by recrystallization from ethyl acetate: yield, 1.9 g; mp 174~176°C. *Anal.* Found: C, 50.02; H, 5.81; N, 12.66. Calcd. for $C_{13}H_{19}N \cdot C_6H_3N_3O_7$: C, 50.21; H, 5.77; N, 12.33%. UV $\lambda_{max}^{MeOH}nm(\epsilon)$: 310 (57,250).

The hydrochloride was then prepared from the picrate. Benzene (400 ml) and 1.0 N sodium hydroxide (160 ml) were added to the picrate (800 mg) and stirred vigorously. After benzene layer was dehydrated, dried hydro-

chloric acid gas was introduced into the benzene solution. White crystals (pyrindicin hydrochloride) were obtained from the concentrate of the benzene solution: yield 370 mg; 145°C (decomp.). *Anal.* Found: C, 68.94; H, 8.82; N, 5.98. Calcd. for $C_{13}H_{19}N \cdot HCl$: C, 69.16; H, 8.92; N, 6.20%. UV $\lambda_{max}^{MeOH}nm(\epsilon)$: 306 (33,250). $[\alpha]_D^{20} + 22.92^\circ$ ($c=0.7$, H_2O). MS m/e : 189.151 (M^+), 174.128 ($M^+ - CH_3$), 160.112 ($M^+ - C_2H_5$, base peak). The UV and IR spectra are shown in Figs. 2 and 3, respectively.

FIG. 2. UV-Spectrum of Pyrindicin Hydrochloride (in MeOH).

FIG. 3. IR-Spectrum of Pyrindicin Hydrochloride (KBr).

The compound, pyrindicin hydrochloride, was freely soluble in chloroform, methanol and water, but insoluble in benzene, ethyl acetate and ethyl ether. It gave positive reactions against Dragendorff's, iodoplatinate, Mayer's and Wagner's reagents, but negative against Ehrlich's and ninhydrin reagents.

From the above data, it is evident that the alkaloid is a new compound, differing from

nigrifactin,¹²⁾ arglecin,^{21,22)} dienomycin,²³⁾ sphydrofuran^{24,25)} and antipain²⁶⁾ obtained by chemical screening from actinomycetes strains. The further experiments by M. Onda *et al.*¹⁵⁾ revealed that this compound is a new 1-pyrindine derivative having the structure of 5-(*trans*-1'-butenyl)-3-methyl-3,4,6,7-tetrahydro-2*H*-1-pyrindine.

V. Biological activities of pyrindicin

The minimal growth inhibitory concentration of pyrindicin hydrochloride was determined according to the conventional agar dilution method. The results are listed in Table X. Pyrindicin had a weak antimicrobial

TABLE X. ANTIMICROBIAL SPECTRUM OF PYRINDICIN HYDROCHLORIDE

Test organism	MIC ^{a)} (μg/ml)
<i>Bacillus subtilis</i> PCI 219	> 500
<i>Staphylococcus aureus</i> FDA 209P	> 500
<i>Sarcina lutea</i> PCI 1001	250
<i>Lactobacillus fermenti</i>	> 500
<i>Mycobacterium smegmatis</i> ATCC 607	250
<i>Escherichia coli</i> NIHJ	250
<i>E. coli</i> NIHJ JC-2	500
<i>Saccharomyces sake</i>	> 500
<i>Pseudomonas aeruginosa</i> P-3	> 500
<i>Candida albicans</i>	> 500
<i>Xanthomonas oryzae</i>	± 500
<i>Aspergillus niger</i>	> 500
<i>Trichophyton interdigitale</i>	500

^{a)} Minimal inhibitory concentration assayed by agar dilution method.

activities against *Sarcina lutea*, *Mycobacterium smegmatis* and *Escherichia coli*.

Besides, this compound was found to possess the following pharmacological activities: narcoantagonistic (50 mg/kg, sc, in mice), analgesic (antiwrithing, 25 mg/kg, sc, in mice), intestine relaxation (1 μg/ml, *in vitro*, guinea pig), and the inhibition of platelet aggregation induced by collagen (10 μg/ml, *in vitro*, rabbit).

An antitumor effect of the compound was also examined. From 24 hr after transplantation of mouse leukemia on CDF₁ mice, 1 to 8 mg/kg of the compound was injected intraperitoneally once a day for 10 days. The

survival effect was not observed.

The acute toxicity (LD₅₀) of the compound, pyrindicin hydrochloride, by intraperitoneal administration in mice was 87 mg/kg.

DISCUSSION

In this paper, we have demonstrated that a new alkaloid, pyrindicin, was produced by a new *Streptomyces* strain, *S. griseoflavus* var. *pyrindicus* nov. var.

It is an interesting problem that the compound has several pharmacological activities. Further investigation for the pharmacological activities is now in progress.

The other alkaloidal substances produced by *Streptomyces* strains, which were selected by our screening research, are also under investigation at present.

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