NOVEL ANTITUMOR ANTIBIOTIC PHOSPHOLINE

1. PRODUCTION, ISOLATION AND CHARACTERIZATION

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Phospholine was isolated as an antitumor antibiotic from the fermentation broth of *Streptomyces hygroscopicus*. Phospholine is an amphoteric compound which has an amino group and a phosphoric acid ester as functional groups. Phospholine shows strong activities against L1210, P388 and EL-4.

In our screening program to find antitumor antibiotics, strain SCM-127 indentified as *Streptomyces hygroscopicus* subsp. *luteolus* subsp. nov. was found to produce a new antitumor antibiotic, phospholine. In this paper, the taxonomy of the producing strain, isolation physico-chemical and biological properties of phospholine are described.

Materials and Methods

Chemicals

Chemicals employed are as follows; Packed column of ODS from Yamamura Scientific Co., Ltd., Kyoto, Japan. Silica gel and TLC-plate Silica gel 60 F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG. All other chemicals are of analytical grade.

Assay

In vitro cytotoxicity assay was carried out against L1210 murine leukemia cells, P388 murine leukemia cells and EL-4 murine lymphoma cells. Each cells in RPMI-1640 medium containing 10% fetal calf serum and the test sample solutions were planted to the wells and incubated at 37°C for 72 hours with 5% CO_2 under high humidity condition. The cytotoxicity of the test samples was determined by counting viable cells after staining with trypan blue. The percent inhibition was calculated by the formula, $((A-B)\times 100/A)$, where A is the number of viable cells without the drug and B is that with the drug.

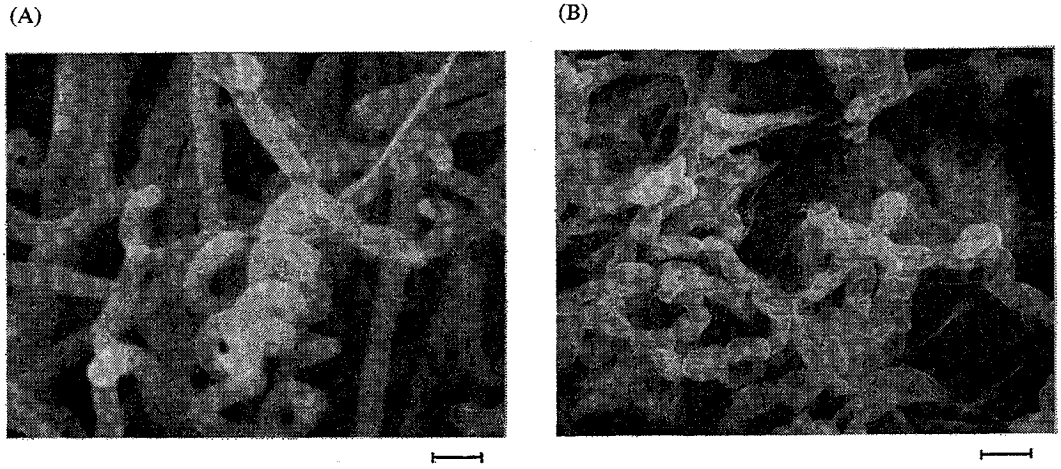
Taxonomy

A soil sample was collected at Katsuta-city, Ibaragi Prefecture, Japan. The taxonomic studies were carried out by the methods of International Streptomyces Project (ISP)[1], along with several supplementary tests. Stock slant cultures were maintained on BENNETT's agar. These slants, as well as subsequent cultures used in this study, were incubated at 28°C. Microscopic observations were made on cultures that were grown for 7~21 days on sucrose - nitrate agar, glycerol - asparagine agar, inorganic salts - starch agar, yeast extract - malt extract agar, and oatmeal agar media.

Taxonomic keys of BERGEY's Manual of Determinative Bacteriology (8th Ed.), Actinomycete Taxonomy[2] and other descriptions were used to compare with recognized genera and species of actinomycetes. Chemotaxonomic studies were carried out by the method of LECHEVALIER and

Fig. 1. Scanning electron micrograph of aerial mycelia of strain SCM-127.

Cultured on inorganic salts - starch agar incubated at 28°C for (A) 10 days and (B) 21 days. Bar represents 1 μm.



LECHEVALIER[3]. Light and electron microscopic studies showed that fairly long, straight and flexous aerial mycelia were formed from the branched vegetative mycelia grown on both synthetic and complex media. Sporophores were arranged singly, in pairs or occasionally in tufts along the straight or flexous main aerial mycelia, terminating in coil. Mature spore chains contained more than 10 spores per chain. The spore shape was short-cylindrical or elliptical ($0.5\sim0.6\times0.8\sim1.5$ μm) and the spore surface was smooth (Fig. 1). Whirls and other special morphologies such as sporangia, zoospores, ball like bodies or sclerotia were not observed.

On most media, the aerial mycelium was abundant and varies from light gray to brownish gray in mass color. After 2 or 3 weeks, moist black, liquefied (hygroscopic) areas were also found in the aerial mycelium. This phenomenon was common in yeast extract - malt extract agar, oatmeal agar, inorganic salts - starch agar. The color of vegetative mycelium was pale yellow to pale brown when grown on yeast extract - malt extract agar, inorganic salts - starch agar, glycerol - asparagine agar, nutrient agar and BENNETT's agar. Soluble pigment was not produced. Results are shown in Table 1. Physiological properties of the strain are summarized in Table 2. Nitrate reduction, starch hydrolysis, milk peptonization and milk coagulation were positive, whereas gelatin liquefaction and melanoid pigment production, tyrosinase reaction, H_2S production, and cellulolytic activity was nagative. Temperature range for growth was from $15\sim33$°C with the optimum in a range of $24\sim27$°C. NaCl tolerance was less than 12% on both nutrient agar and BENNETT's agar. Utilization of carbohydrate by the strain was shown in Table 3.

The cell wall analysis of strain SCM-127 showed that it contained LL-diaminopimelic acid and glycine. Accordingly, the cell wall of the strain was Type 1.

The morphological, cultural and physiological characteristics of strain SCM-127, described above, revealed that the strain was classified in the genus *Streptomyces* Waksman and Henrici 1943 339. Accordingly, the strain was compared with the published descriptions[4~9] of various *Streptomyces* species and the results showed that strain SCM-127 was considered to resemble *S. hygroscopicus*, *S. hygroscopicus* var. *angustmyceticus*[5], *Streptomyces platensis*[8] and *Streptomyces neohygroscopicus*

Table 1. Cultural characteristics of strain SCM-127 on various media.

Yeast extract - malt extract agar (ISP No. 2)	G: Moderate, pale yellowish brown R: Pale yellow AM: Moderate, brownish gray SP: None
Oatmeal agar (ISP No. 3)	G: Abundant, pale yellowish brown R: Yellowish brown AM: Abundant, brownish gray SP: None
Inorganic salts - starch agar (ISP No. 4)	G: Abundant, pale yellowish brown R: Pale yellow AM: Abundant, brownish gray SP: None
Glycerol - asparagine agar (ISP No. 5)	G: Moderate, pale yellowish brown R: Pale yellow AM: Moderate, Brownish gray SP: None
Tyrosine agar (ISP No. 7)	G: Abundant, yellowish brown R: Pale yellow AM: Abundant, light gray SP: None
Sucrose - nitrate agar	G: Abundant, pale yellowish brown R: Pale brown AM: Moderate, grayish yellow SP: None
Nutrient agar	G: Moderate, pale yellow R: Pale yellow AM: Thin, brownish gray SP: None
BENNETT's agar	G: Abundant, pale yellowish brown R: Pale yellow AM: Moderate, dark brownish gray SP: None

Abbreviations: G, Growth of vegetative mycelium; R, reverse side of color; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain SCM-127.

Melanoid pigment production	−
Tyrosinase reaction	−
H_2S production	−
Gelatin liquefaction (21°C)	−
Milk peptonization (37°C)	+
Milk coagulation (37°C)	+
Nitrate reduction	+
Cellulolytic activity	−
Starch hydrolysis	+
Temperature range for growth	15~33°C
Optimum temperature for growth	24~27°C
NaCl tolerance	≦12%

+: Positive, −: negative.

Table 3. Utilization of carbon sources by strain SCM-127.

D-Glucose	+
L-Arabinose	±
D-Fructose	+
D-Xylose	+
D-Mannitol	+
Sucrose	+
L-Rhamnose	−
Raffinose	+
Inositol	+

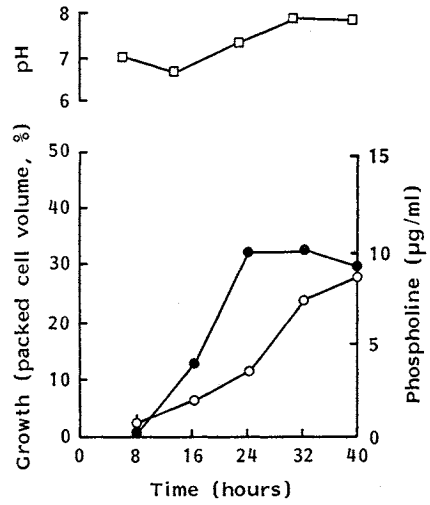
+: Positive, ±: weakly positive, −: negative.

subsp. *globomyceticus*[9]. DIETZ proposed that *S. hygroscopicus* with smooth spore surface belonged to *S. neohygroscopicus*[4]. *S. hygroscopicus* var. *angustmyceticus*, *S. platensis* and *S. neohygroscopicus* subsp. *globomyceticus* are involved in this category. However, these strains were clearly distinguished from strain SCM-127 by their cultural characteristics, physiological properties. Therefore, the cultural characteristics of strain was directly compared with *S. hygroscopicus* ATCC 13810. Strain SCM-127 differed from *S. hygroscopicus* on the ability of milk coagulation, gelatin liquefaction and the utilization of L-rhamnose, sucrose and raffinose. In addition, the color of the reverse side of the colony of strain SCM-127 was pale yellow, whereas that of *S. hygroscopicus* was pale brown to pale yellowish brown. As a result, strain SCM-127 is considered to be a new subspecies of *S. hygroscopicus* and the name *S. hygroscopicus* subsp. *luteolus* subsp. nov. (lu. te'o. lus. L. adj. *luteus* yellow; L. dim. adj. *luteolus* somewhat yellow, referring to the color of the reverse side of the colony) is proposed. The type strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with accession No. FERM P-8822.

Fermentation

Strain SCM-127 was grown on BENNETT's agar slants. Slants were incubated at 28°C for 10 to 14 days and then stored at 15°C until needed. A loopful of the slant culture of strain SCM-127 was inoculated into a seed medium (60 ml) containing glucose 0.5%, glycerol 1.0%, dextrin 2.0%, soybean meal 0.5%, meat extract 0.5%, $CaCO_3$ 0.4% (pH 7.0 before sterilization) in 500-ml Erlenmeyer flasks and cultured at 28°C on a rotary shaker at 200 rpm for 72 hours. The seed culture was transferred at the rate of 2.0% to 20 liters of production medium in a 30-liter jar fermenter and cultivation was carried out for 40 hours at 28°C under aeration of 30 liters/minute, agitation of 300 rpm and inner pressure of 0.5 kg/cm^2. The production medium consisted of: Dextrin 1.0%, D-mannose 1.0%, soybean meal 1.0%, cotton seed meal 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05% and Adecanol (Asahi Denka Kogyo Co., Ltd.) 0.03% (pH 7.0 before sterilization). A typical time course of phospholine fermentation in a 30-liter jar fermenter at 28°C for 40 hours is shown in Fig. 2. Production of phospholine started at 8 to 16 hours after inoculation, then gradually increased and reached at maximum (10 μg/ml).

Fig. 2. Time course of phospholine production.
□ pH, ○ growth, ● phospholine.



Isolation

After completing the fermentation, the culture broth (85 liters) was adjusted to pH 7.0 with 4 N HCl and filtered with an aid of Celite (4 kg). The filtrate was adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Industries Co., Ltd.) (7.2 liters). The column was washed with water and eluted with 50% aqueous acetone (22 liters). The eluate was concentrated to 6 liters and then the concentrated solution was extracted with ethyl acetate. To the aqueous layer was added buthanol and extracted. After washing with water, the buthanol layer was

Fig. 3. IR spectrum of phospholine.

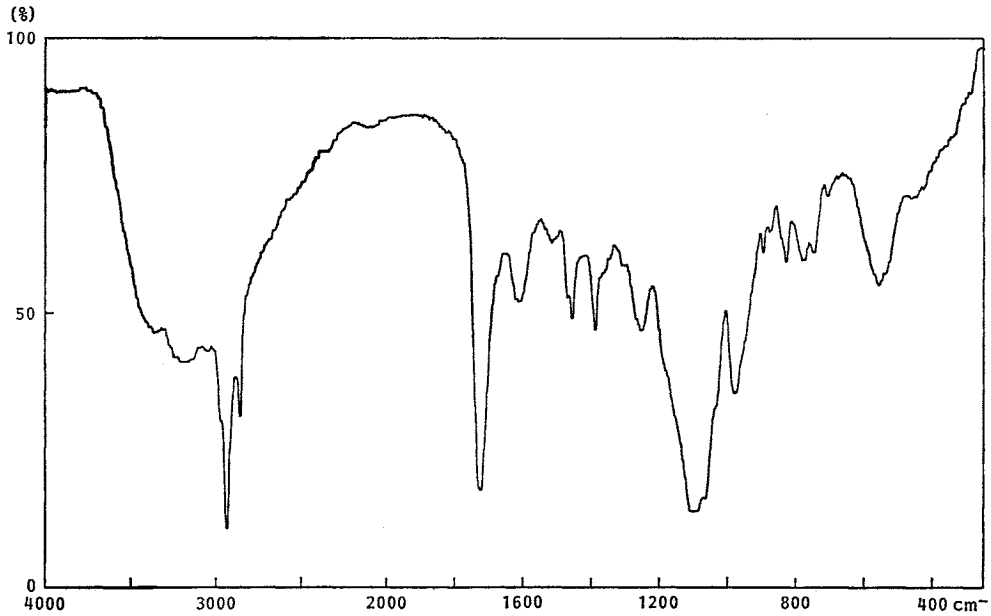
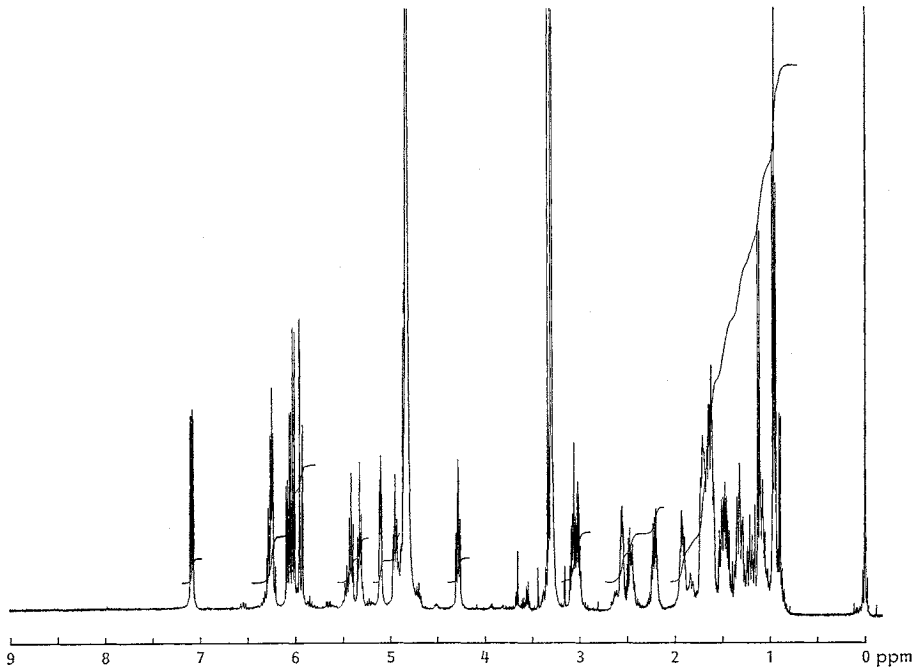


Fig. 4. ^{1}H NMR spectrum of phospholine.



concentrated *in vacuo* to give an oily residue. The residue was dissolved in a small amount of propanol - water (5 : 1) and applied to a silica gel column (Wakogel C-200, Wako Pure Chemical Industries, Ltd.) (240 ml) packed with the same solvent. The column was eluted with propanol - water (5 : 1). The eluate was monitored by both the cytotoxicity against HeLa cells and the silica gel

Fig. 5. ^{13}C NMR spectrum of phospholine.

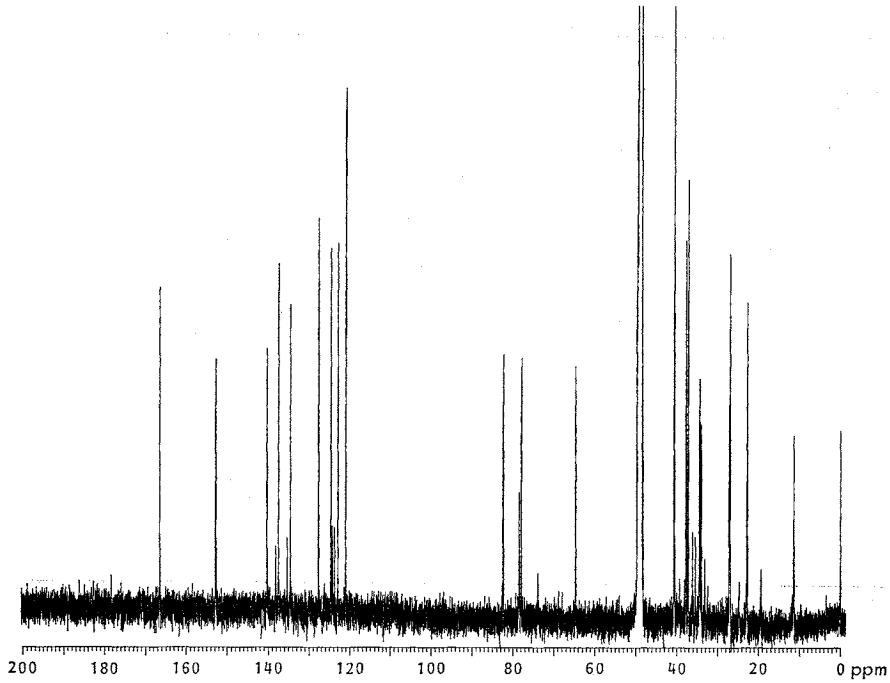


Table 4. Physico-chemical properties phospholine.

Nature	White powder
MP (degradation)	153~158°C
$[\alpha]_D^{21}$ (*c* 1.0, MeOH)	+81°
Elementary analysis (as sodium salt)	
Calcd for $C_{25}H_{39}NO_8PNa \cdot 2H_2O$:	C 52.53, H 7.58, N 2.45, P 5.42
Found:	C 52.13, H 7.26, N 2.50, P 5.14
FAB-MS (*m/z*)	514 (M+1), 536 (M+Na)
Rf value[a]	0.38 (solvent 1)
	0.31 (solvent 2)

[a] Kieselgel 60 F_{254} (E. Merck).
Solvent 1: Acetonitrile - water (5 : 2).
Solvent 2: Propanol - water (3 : 1).

chromatography (Kieselgel 60 F_{254}, E. Merck; acetonitrile - water (5 : 1)). The active fractions were collected and concentrated *in vacuo* to give a light brown residue. The residue was further chromatographed on silica gel column (40 ml) (Kieselgel 60 F_{254}) using CH_3CN - MeOH - H_2O - 2-PrOH (9 : 4 : 3 : 1) as a solvent. The active fractions were collected and again chromatographed on preparative thin-layer plates (Kieselgel 60 F_{254}) using CH_3CN - H_2O (5 : 1) as a development solvent. A fraction containing phospholine was collected and extracted with the same solvent. The extract was concentrated *in vacuo* to dryness. The residue was applied to HPLC (ODS; 10 i.d. × 250 mm) and eluted with tetrahydrofuran - acetonitrile - 0.02 M phosphate buffer (pH 7.0) (10 : 20 : 70). Fractions containing phospholine was collected and concentrated to a small volume. To desalt, the concentrated solution was adsorbed on Diaion HP-20 column (5 ml) and eluted with 50% aqueous acetone after washing with water. The eluate was concentrated *in vacuo* to give phospholine (9 mg) as a white powder.

Physico-chemical Properties

Phospholine is soluble in methanol, dimethyl sulfoxide, *N,N*-dimethylformamide, but practically insoluble in ethyl acetate and hexane. It shows positive responses to ninhydrin and ammonium molybdate - perchloric acid and a negative response to Fehling reagent. The molecular formula of phospholine was determined to be $C_{25}H_{40}NO_8P$ by high-resolution fast atom bombardment (HRFAB-MS), elemental analysis and ^{13}C-^{1}H correlation spectroscopy NMR spectrum. The UV spectrum shows an absorption at 234 nm in methanol. The IR spectrum is shown in Fig. 3. The ^{1}H and ^{13}C NMR spectra are shown in Figs. 4 and 5, respectively. Physico-chemical properties of phospholine are summarized in Table 4.

Table 5. Cytotoxicity of phospholine against various tumor cells.

Cell lines	Cytotoxicity (IC_{50}, μg/ml)	
	Phospholine	Mitomycin C
L1210	3.37	0.066
P388	2.00	0.037
EL-4	1.99	0.023

Biological Properties

Phospholine was tested for *in vitro* cytotoxicity against L1210, P388 and EL-4. The cytotoxicity of phospholine is shown in Table 5.

Discussion

Phospholine is a new antitumor antibiotic isolated from the culture broth of *Streptomyces*. A phospholine has an α,β-unsaturated δ-lactone as a functional group from the IR and NMR spectra. There are leptomycin[10,11], kazusamycin[12] and PD 124,895[13] as antibiotics that have a α,β-unsaturated δ-lactone. These antibiotics are different from phospholine which is amphoteric; phospholine has a amino group and a phosphoric group. STAMPWARA *et al.*[14] reported an isolation of CI-920 which has both α,β-unsaturated δ-lactone and phosphoric group. The structure of CI-920 was decided by HOKANSON and FRENCH[15]. CI-920 does not have an amino group. Related antibiotics, MA-5000[16] and AF-273[17], are reported by two groups.

Acknowledgments

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