ISOLATION AND CHARACTERIZATION OF PHOSMIDOSINE A NEW ANTIFUNGAL NUCLEOTIDE ANTIBIOTIC

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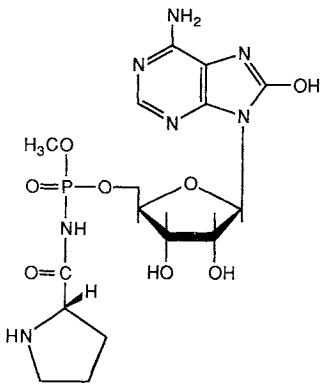
A new nucleotide antibiotic, phosmidosine was isolated from a culture filtrate of a newly isolated streptomycete identified as *Streptomyces* sp. RK-16. HRFAB-MS and elemental analysis established the molecular formula of $C_{16}H_{24}N_7O_8P$. ^1H, ^{13}C and ^{31}P NMR indicated the presence of a methyl phosphate group and UV spectra were similar to those of 8-hydroxyadenosine. The antibiotic inhibited spore formation of *Botrytis cinerea* at the concentration of 0.25 μg/ml.

In the course of our screening for antibiotics active against phytopathogenic fungi, a streptomycete, isolated from a soil sample collected in Wakayama Prefecture, Japan, was found to produce a new antifungal antibiotic. The compound showed specific inhibitory activity against the spore formation of *Botrytis cinerea*, (*Botryotinia fuckeliana*) which is a world-wide pathogenic fungus causative of a gray mold disease of a variety of fruits and vegetables. This compound is shown to be a new nucleotide and designated phosmidosine (Fig. 1). This paper is concerned with taxonomic studies of the producing strain, fermentation, isolation, physico-chemical and biological properties of phosmidosine. Structural elucidation will be reported in a separate paper[1].

Fig. 1. Structure of phosmidosine.



Taxonomic Studies

The producing actinomycete, strain RK-16, was cultured on various agar media and the cultural characteristics are summarized in Table 1. The strain grew well on media of starch-yeast extract agar, yeast extract-malt extract agar, inorganic salts-starch agar, tyrosine agar, and sucrose-nitrate agar. Melanoid pigment was produced on tyrosine agar and peptone-yeast extract-iron agar media, but soluble pigment was not detected on other media. Table 2 shows the physiological characteristics of the strain RK-16. It utilized most of carbohydrates tested except D-fructose and sorbitol.

The spore chains of the strain formed long spirals and the spores are oval with an average diameter of 0.75 × 0.9 μm. The spore surface was spiny (Fig. 2). L,L-2,6-Diaminopimelic acid was detected in whole

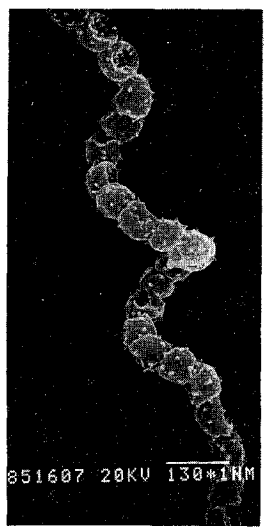
Table 1. Cultural characteristics of strain RK-16.

Medium	Growth	Reverse color	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	Good	Golden brown (3 pg)	Abundant Light gray (c)	None
Oatmeal agar (ISP No. 3)	Poor	Light gray (c)	Poor Gray (f)	None
Inorganic salts-starch agar (ISP No. 4)	Good	Bright yellow (1a)+gray (b)	Poor	None
Glycerol-asparagine agar (ISP No. 5)	Good	Cream (ca)+white (a)	Moderate Cream (ca)+light gray (c)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	Antique gold (pe)	Poor	Melanoid
Tyrosine agar (ISP No. 7)	Good	Black (p)	Poor	Melanoid
Starch-yeast extract agar	Good	Dark brown (3 nl)	Abundant Light brown (3 lg)+gray (g)	None
Nutrient agar	Poor	Gold (1c)	Poor	None
Glucose-asparagine agar	Moderate	Cream (ca)+light gray (c)	Poor Cream (ca)+light gray (c)	None
Sucrose-nitrate agar	Poor	White (a)	Poor Light gray (c)	None

Table 2. Physiological properties and carbon utilization of strain RK-16.

Growth temperature range	23~37°C	Utilization of carbon sources:			
Cell wall constituent	L,L-DAP	L-Arabinose	++	Lactose	++
Hydrolysis of starch	Positive	D-Xylose	++	Sucrose	+
Liquefaction of gelatin	Negative	L-Rhamnose	++	Maltose	+
Peptonization of milk	Weak	D-Glucose	++	Melibiose	++
Formation of melanin	Positive	D-Fructose	−	Raffinose	++
		D-Mannose	++	Sorbitol	−
		D-Galactose	++	Inositol	+
		L-Sorbose	−	Salicin	+

Fig. 2. Electron micrograph of strain RK-16.



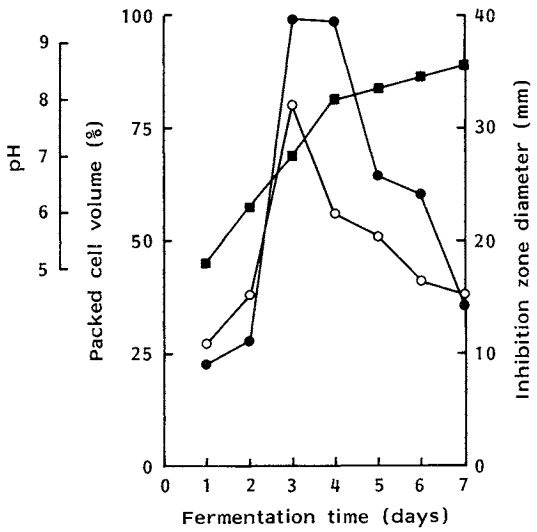
cell hydrolysates. Based on morphological and chemical analyses, the producing culture was identified as a member of the genus *Streptomyces*. It has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan and assigned accession number FERM-P9196.

Fermentation

The time course of growth and antibiotic production of the strain in a flask culture are shown in Fig. 3. The production of phosmidosine reached maximum between three and four days. After 4 days titers decreased remarkably. In case of large scale cultivation, jar fermentation was carried out with a 30-liter jar fermenter containing 18 liter of culture

Fig. 3. Profile of phosmidosine fermentation.

○ Packed cell volume, ■ pH value, ● antifungal activity against *Botryotinia fuckeliana*.



medium for 72 hours at 28°C.

Isolation

Phosmidosine was extracted from the culture filtrate with the cation exchange resin IRC-50 (H^+ form). Isolation procedure is described in the Experimental part. The activity was monitored by a conventional paper disc method using *B. fuckeliana* IFO 5365 as a test organism. Finally the antibiotic was purified by preparative HPLC using a reversed phase column. The isolation yield from fermentation broth was poor (less than 1%) due to the instability of phosmidosine.

Characterization

Phosmidosine is a colorless powder which is soluble in water, slightly soluble in methanol and insoluble in most of organic solvents. Its physico-chemical properties are listed in Table 3. The molecular formula was established as $C_{16}H_{24}N_7O_8P$ based on elemental analysis and HRFAB-MS. The presence of a phosphorous atom in the molecule was supported by the ^{31}P NMR spectrum (δ_p 10.5 ppm in D_2O, H_3PO_4 as a standard). It showed positive color reactions to ninhydrin and Lemieux reagents. It exhibited characteristic UV maxima at 255 (ε 12,300) and 269 nm (ε 14,200) in neutral aqueous solution, 264 (ε 12,300) and 280 nm (ε 11,350) in 0.1 N hydrochrolic acid and 280 nm (ε 15,600) in 0.1 N sodium hydroxide as illustrated in Fig. 4. The IR, ^1H and ^{13}C NMR spectra are shown in Figs. 5, 6 and 7, respectively.

Phosmidosine is unstable in alkali. When the antibiotic was allowed to stand at room temperature

Table 3. Physico-chemical properties of phosmidosine.

MP	>230°C (dec)
$[\alpha]_D^{20}$ (*c* 1, H_2O)	−19.6°
Molecular formula	$C_{16}H_{24}N_7O_8P$
MW	473
Elemental analysis	
Calcd for $C_{16}H_{24}N_7O_8P \cdot H_2O$:	C 39.10, H 5.29, N 19.95
Found:	C 39.22, H 4.90, N 19.94
HRFAB-MS (*m/z*)	
Calcd for $C_{16}H_{25}N_7O_8P$:	474.1502 (MH^+)
Found:	474.1493 (MH^+)
pKa'	4.4, 8.9
IR ν_{max} (KBr) cm^{-1}	3400, 1720, 1660, 1590, 1400, 1040

Fig. 4. UV spectra of phosmidosine.

--- 0.1 N Hydrochloric acid, ——— distilled water, — 0.1 N sodium hydroxide.

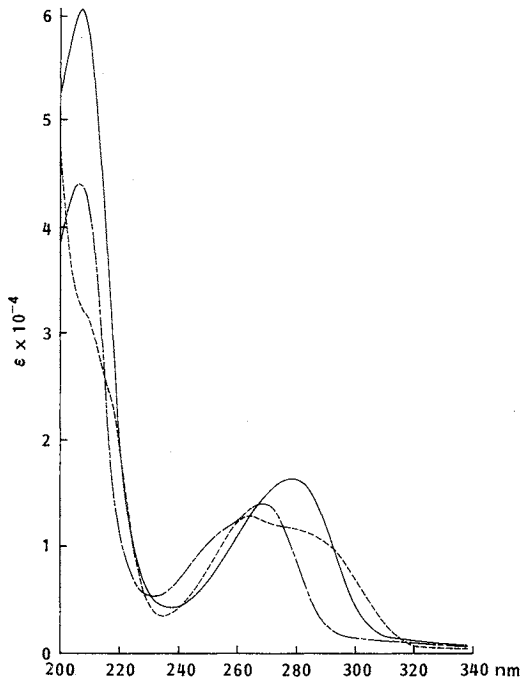


Fig. 5. IR spectrum of phosmidosine (KBr).

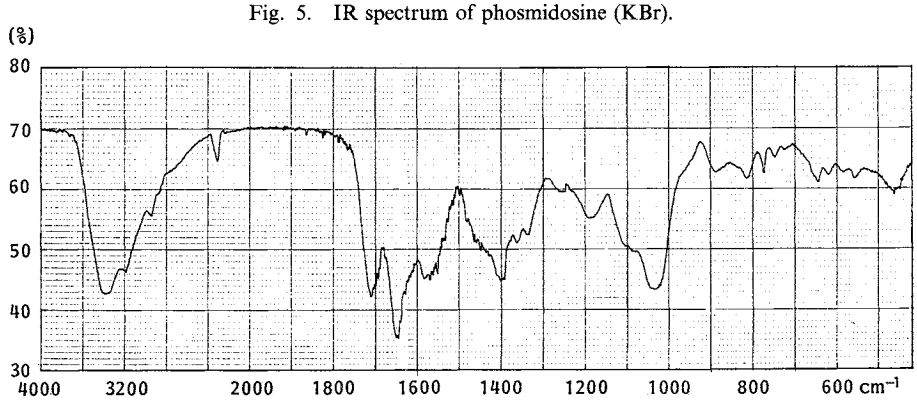


Fig. 6. ^{1}H NMR spectrum of phosmidosine (500 MHz, D_2O).

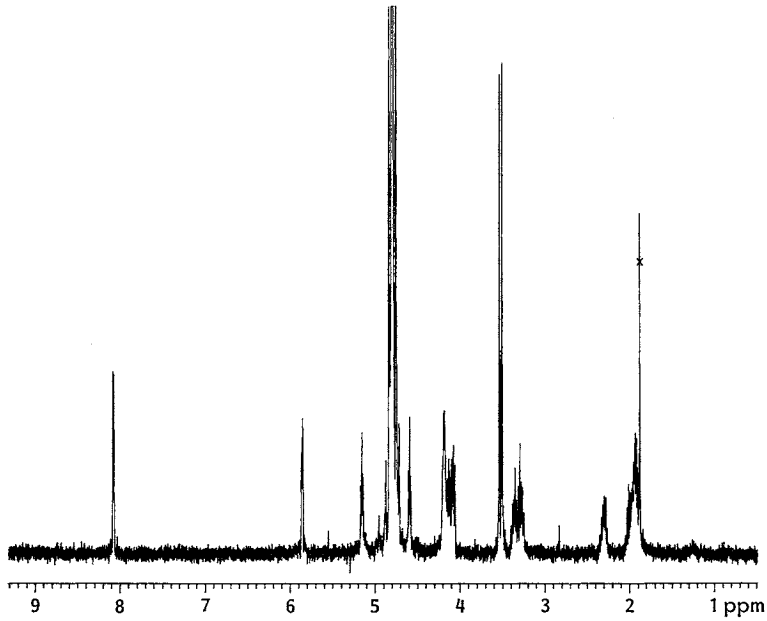


Fig. 7. ^{13}C NMR spectrum of phosmidosine (25 MHz, D_2O).

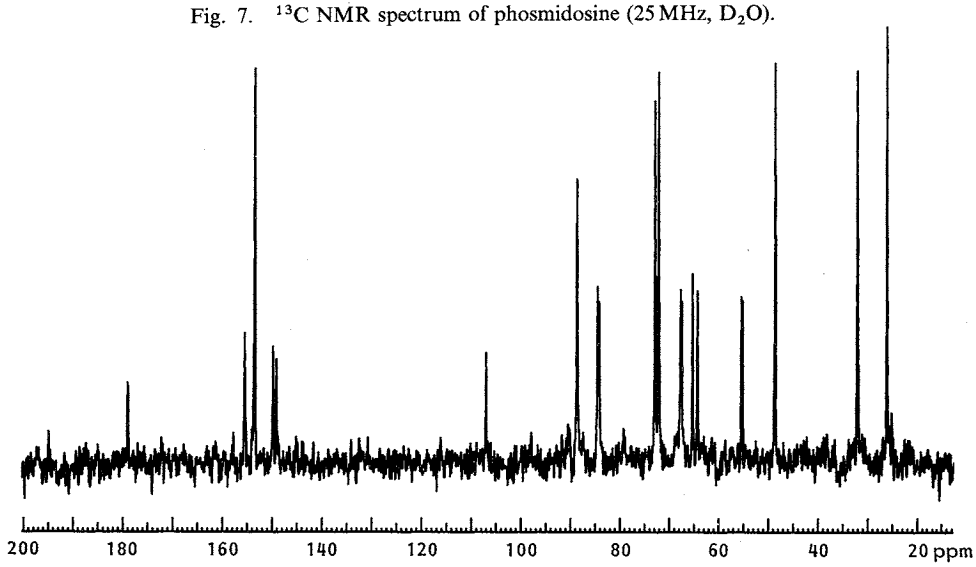
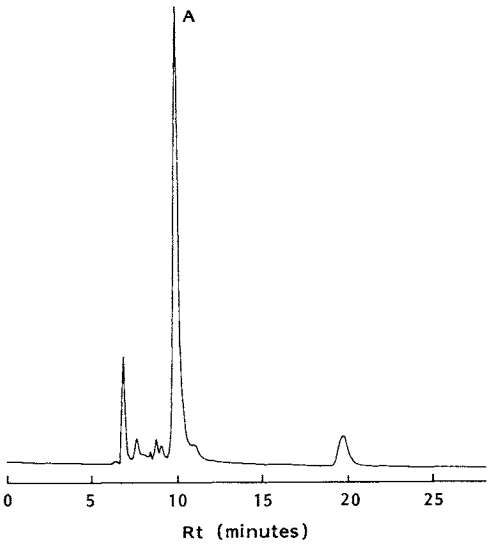


Fig. 8. HPLC profile of phosmidosine isolated by preparative HPLC.



Column: Senshu Pak ODS-H-4251. Solvent: 0.004 M citrate buffer - methanol (75 : 25). Monitor: 270 nm. Flow rate: 1.5 ml/minute. Peak A represents phosmidosine. Other peaks are degradation products.

Table 4. Inhibition of spore formation of fungi by phosmidosine.

Test organism	MIC for spore formation (μg/ml)[a]
Botryotinia fuckeliana IFO 5365	0.25
Aspergillus niger JCM 5697	1.0
Alternaria mali IFO 8984	>500
Colletotrichum lagenarium IFO 7513	>500
Pyricularia oryzae IFO 5994	>500

[a] Assayed by dilution method on potato - sucrose agar and spore formation was observed under a microscope after incubation at 27°C for 72 hours.

in alkaline solution (pH 10) for 2 days, the inhibitory activity against spore formation of *B. fuckeliana* decreased by 60%. Heating phosmidosine at 100°C for 5 minutes at pH 10 resulted in a loss of 90% of the original activity and at pH 6 in a loss of 60%. Under acidic conditions, it is relatively stable. 20% of the activity was lost in pH 2 solution after treatment at 100°C for 5 minutes.

Following treatment of phosmidosine with 0.3 N ammonium hydroxide at room temperature, several peaks were observed by HPLC. These peaks appeared to be degradation products. After preparative HPLC, rechromatography of the resulting solution showed a single peak. However, after desalting with Dowex 50 (H^+), the resulting lyophillized powder exhibited several peaks due to degradation products by HPLC analysis (Fig. 8).

Biological Properties

Phosmidosine showed specific spore - formation inhibitory activity against *B. cinerea* (*B. fuckeliana* IFO 5365) and *Aspergillus niger* JCM 5697, but was not active against most of the other fungi, bacteria and yeasts tested. On treatment with phosmidosine, mycelial growth of *B. fuckeliana* was not inhibited, but spore formation, as observed under a microscope, was inhibited. The same phenomenon was observed with *A. niger*, the inhibition occurring at the higher concentrations of the antibiotic (Table 4).

Discussion

Based on taxonomic studies of the producing strain, RK-16 (FERM-P9196), it is concluded that it belongs to the genus *Streptomyces*. Further studies are necessary for species identification.

Phosmidosine is labile to alkali and it was difficult to obtain the antibiotic in pure form, which explains the low isolation yield (less than 1%) from the fermentation broth.

The presence of a methyl phosphate group in phosmidosine was evidenced by the chemical shift of phosphorus in the ^{31}P NMR spectrum and a doublet of methoxyl methyl protons in the ^{1}H NMR spectrum (δ_H 3.48, d, $J_{H\text{-}O\text{-}P}=11.4$ Hz). It was suggested that the antibiotic had a sugar moiety (δ_H 5.81, d, $J=4.8$ Hz, anomeric-H) and a base part (δ_H 8.01, s). Two of four doublets of ^{13}C signals coupled with phosphorus, were assigned as sugar carbons (δ_C 67.7 and 84.4) and one as a methoxyl carbon (δ_C 55.4). These C-P couplings were confirmed by the consistency of the J values in measuring at 25 MHz and 100 MHz. These

data suggested that phosmidosine is a nucleotide. Only four nucleotide antibiotics, agrocin 84 produced by *Agrobacterium radiobacter*[2], thuringiensin by *Bacillus thuringiensis*[3], fosfadecin by *Pseudomonas viridiflava*[4], and fosfocytocin by *Pseudomonas fluorescens*[5] have been reported. The UV absorption spectra of these antibiotics are quite different from that of phosmidosine, which resembles that of 8-hydroxyadenosine[5]. Therefore, it was concluded[1] that phosmidosine is a new antifungal nucleotide antibiotic.

Experimental

General

The mp was taken on a Yanagimoto micro melting point apparatus and was uncorrected. HPLC was carried out using Hitachi 635A and Waters Lambda-Max model 481 liquid chromatographs. Hitachi 220A and a Shimadzu IR-27G recording IR spectrophotometers were used for measuring the UV and IR spectra, respectively. Optical rotation was measured by a Perkin-Elmer 241MC polarimeter. The NMR spectra were obtained on the following FT NMR spectrometers: a Jeol JNM FX-100 for ^{13}C, a Jeol JNM GX-400 for ^{31}P and ^{1}H and a Jeol JNM GSX-500S for ^{1}H. Chemical shifts were expressed as δ values in ppm downfield from TSP in D_2O as an external standard. The HRFAB mass spectra were obtained uisng a VG 70-SEQ mass spectrometer.

Taxonomic Studies

Taxonomic studies were achieved by using the methods and media described in International Streptomyces Project (ISP)[6]. The cultural characteristics were observed after 7, 14 and 21 days cultivation at 27°C. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) was used as a standard for the comparison of the color of mycelia and soluble pigments. Diaminopimelic acid isomers were analyzed by the method of BECKER *et al.*[7]. The electron micrograph of the strain RK-16 was taken on a Hitachi model S-430 Scanning Electron Microscope.

Fermentation and Isolation

For seed culture, spores of the strain RK-16 from an agar slant were inoculated in a 500-ml cylindrical flask containing 70 ml of medium composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soy bean flour 2.5%, sodium chloride 0.2%, and dipotassium hydrogen phosphate 0.005%. It was cultivated for 48 hours at 28°C on a rotary shaker. Each 2 ml-portion of the culture broth was transferred into flasks containing 70 ml of the same medium and they were incubated at 28°C. For large scale fermentation, the culture broth of the two flasks after 48 hours cultivation was inoculated into a 30-liter jar fermenter containing 18 liters of the same medium, which was fermented for 72 hours at 28°C under aeration of 11 liters/minute and agitation at 350 rpm.

Culture broth (60 liters) was filtered with Celite and the filtrate was passed through a column of IRC-50 (H^+ form). After washing with water, the active component was eluted with 0.5 N ammonium hydroxide and the eluate was immediately neutralized with 3 N hydrochloric acid. The solution was applied to a column of Diaion HP-20. After washing with water, the antibiotic was eluted with 70% aqueous methanol. The eluate was concentrated *in vacuo* to a small volume and charged to a column of activated charcoal. It was developed with water followed by 50% aqueous acetone. The active fractions were collected, evaporated to a small volume and lyophilized to give a crude powder (450 mg). It was dissolved in a small amount of a solvent system of butanol-methanol-water (4:1:2) and loaded on a column of cellulose powder, which was developed with the same solvent system. The active fractions were combined, concentrated and lyophilized to give 30 mg of the crude antibiotic. Further purification was carried out by preparative HPLC using a Senshu Pak ODS-H-4251 (10×250 mm) with a solvent of 0.004 M citrate buffer (pH 4.5)-methanol (85:15) monitored at 235 nm. The eluate corresponding to the target peak was evaporated to remove methanol and applied to a column of Dowex 50W-X8 (H^+ form). The antibiotic was eluted with 0.3% ammonium hydroxide and the eluate was lyophilized to give 6 mg of a white powder of phosmidosine.

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