Communications to the Editor

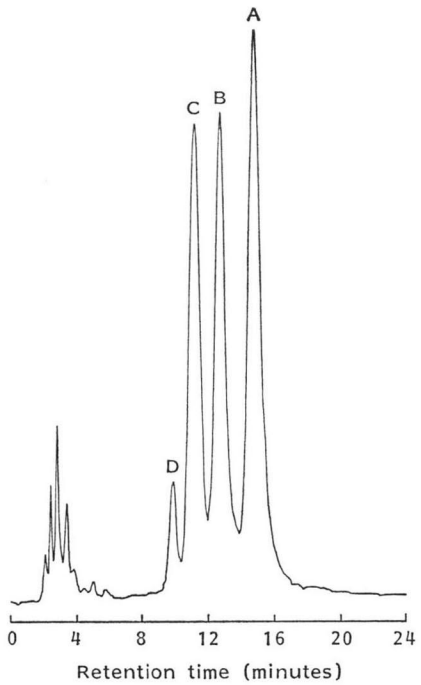
A NEW ANTIFUNGAL ANTIBIOTIC, PHOSPHAZOMYCIN A*

Sir:

In the course of the screening for the inhibitors of fungal cell wall biosynthesis, *Streptomyces* sp. No. RK-803 (FERM-P 6644) isolated from a soil sample collected in Fukushima-shi, Japan, was found to produce a new antibiotic complex active against phytopathogenic fungi. The complex is composed of at least four active components. We wish to describe here isolation and characterization of one of the main active components, phosphazomycin A, which has each one atom of phosphorus and nitrogen in the molecule.

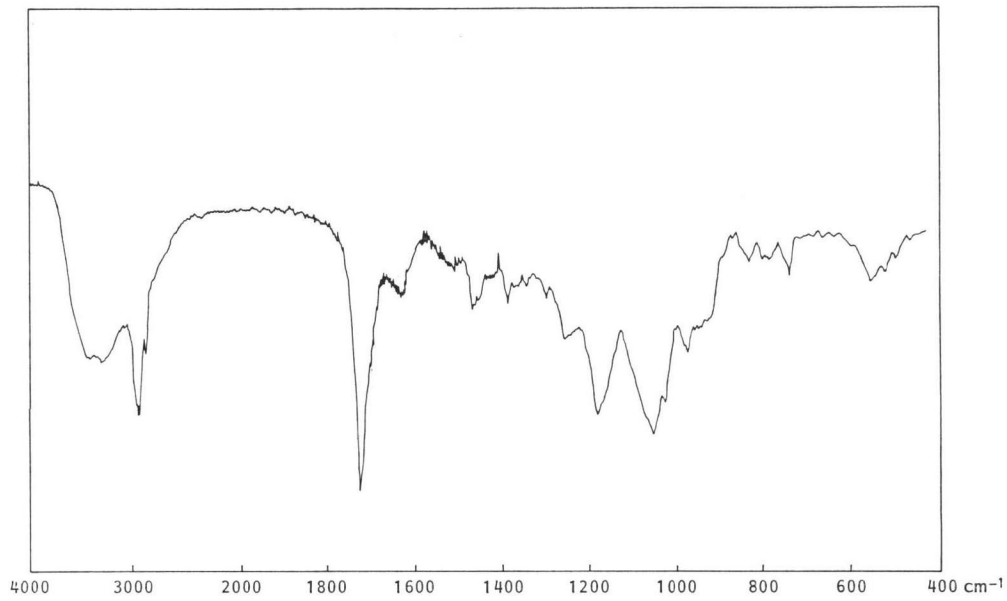
The strain was cultured at 27°C for 72 hours in a jar fermentor containing 18 liters of a medium consisted of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, sodium chloride 0.2%, and dipotassium hydrogen phosphate 0.005%. The culture broth (60 liters) was filtered, and the mycelial cake was extracted with 70% aqueous acetone. After removal of acetone, the aqueous extract was combined with the filtrate, and the mixed solution was passed through a column of Diaion HP-10 (6 liters). After it was washed with water and 40% aqueous methanol, active components were eluted with 50% aqueous acetone. Bioactive eluate was evaporated *in vacuo* to remove acetone. From the resulting aqueous solution, antibiotic complex was extracted with 1-butanol. The organic layer was concentrated to a small volume, to which excess volume of water was added to yield viscous precipitate. Supernatant solution was discarded by decantation, and the residue was dissolved in a small volume of methanol. The solution was passed through a column of activated charcoal, which was washed with water. Bioactive components were fractionated by stepwise-elution with following solvent system in the order; 40% aqueous methanol, 40% and 70% aqueous acetone. Bioassay by agar plate method using *Alternaria mali* as a test microorganism showed that inhibitory activity was observed in extended fractions obtained by elution with 40% to 70% aqueous acetone. Active fractions were collected and concentrated *in vacuo* to afford a crystalline mixture. After filtration, a crude crystalline powder (2 g) was obtained. Crude antibiotic complex (700 mg) was dissolved in a small amount of methanol and subjected to a high performance liquid chromatography (HPLC) using PrepPak-500/C18 (50×300 mm) by Prep LC/System 500A (Waters) with a solvent system of a buffer (1% triethylamine - phosphoric acid, pH 7) - acetonitrile (15: 85), yielding purified phosphazomycin complex (150 mg). Fig. 1 shows the analytical HPLC of the antibiotic complex. Four active components were designated as A, B, C and D.

Fig. 1. HPLC profile of phosphazomycin complex.
Column, Nucleosil $5C_{18}$ (6×250 mm); solvent system, acetonitrile - buffer (1% triethylamine - phosphoric acid, pH 7)=85: 15; detector, UV (235 nm); flow rate, 1 ml/minute.



* Phosphazomycin A is the identical compound with RK-803 reported in the abstract of the papers of the annual meeting of the Agricultural Chemical Society of Japan (1983, p. 115) and HK-803 in Japan 84-31,689, 1984.

Fig. 2. IR spectrum of phosphazomycin A (KBr).



Peak A was separated by the preparative HPLC, and the solution was passed through a column of Diaion HP-10, which was then eluted with 50% aqueous acetone. The eluate was concentrated to a small volume and lyophilized to give a colorless powder of phosphazomycin A. Further purification was carried out by preparative HPLC using Nucleosil $5C_{18}$ (10× 200 mm) with the same solvent system. The single peak fraction was collected and adsorbed on a column of Diaion HP-10, which was eluted by 50% aqueous acetone. After concentration and lyophilization, the residue was recrystallized from ethanol - water affording colorless crystals of phosphazomycin A (20 mg).

Phosphazomycin A [mp 155~158°C with decomposition; $[\alpha]_D^{20}$ +87.8° (*c* 0.6, methanol)] is a weakly acidic compound as judged from a slight mobility toward a cathod at pH 8 (0.1 M Tris-HCl buffer) in high voltage paper electrophoresis (2,500 V, 60 minutes). It has an absorption maximum at 233 nm ($E_{1cm}^{1\%}$ 516) in methanol. The IR and ^1H NMR spectra are shown in Figs. 2 and 3, respectively. Elementary analysis was as follows: C 57.15%, H 7.90%, N 1.98%, P 3.19%. The ^{13}C NMR spectrum (Fig. 4) shows 37 or 38 observable carbon signals. Potentiometric titration of phosphazomycin A showed a *pKa'* of 7.4 with an equivalent weight of 742. On the basis of these data, a tentative molecular formula is proposed to be $C_{37\sim38}H_{56\sim60}O_{12\sim13}NP$ for the antibiotic. It was soluble in methanol, ethanol and alkaline water, slightly soluble in acetone and water, and insoluble in most of the other organic solvents. It was positive to potassium permanganate, anisaldehyde-sulfuric acid, Dittmer-Lester, and ammonium molybdate-perchloric acid reagents.

Chemical shift of ^{31}P signal in the NMR spectrum (δ_P 2.1 ppm, d, J_{P-O-CH}=10 Hz) suggested a P-O-CH group in phosphazomycin A. On treatment of the antibiotic with 0.5 N HCl at 60°C for 4 hours followed by neutralization with potassium carbonate, one equivalent of ammonia was determined by Nessler reagent. Hydrolysis of phosphazomycin A with alkaline phosphatase from calf intestine or 0.5 N HCl afforded phosphoric acid as detected by ammonium molybdate-perchloric acid reagent on TLC plate. These data as well as the acid labile nature of the antibiotic suggested the presence of a phosphonamide group in the molecule. Absorption maximum at 233 nm in the UV spectrum suggested the presence of α,β-unsaturated carbonyl system in phosphazomycin A. This is supported by the IR (ν_{max} 1720 cm^{-1}, unsaturated lactone) and the ^1H NMR spectra (δ 7.09 ppm, 1H, dd, J=5, 10 Hz, β proton of α,β-unsaturated lactone).

Among the known antifungal antibiotics,

Fig. 3. ^{1}H NMR spectrum of phosphazomycin A (400 MHz, CD_3OD, WEFT, τ 2.5 seconds).

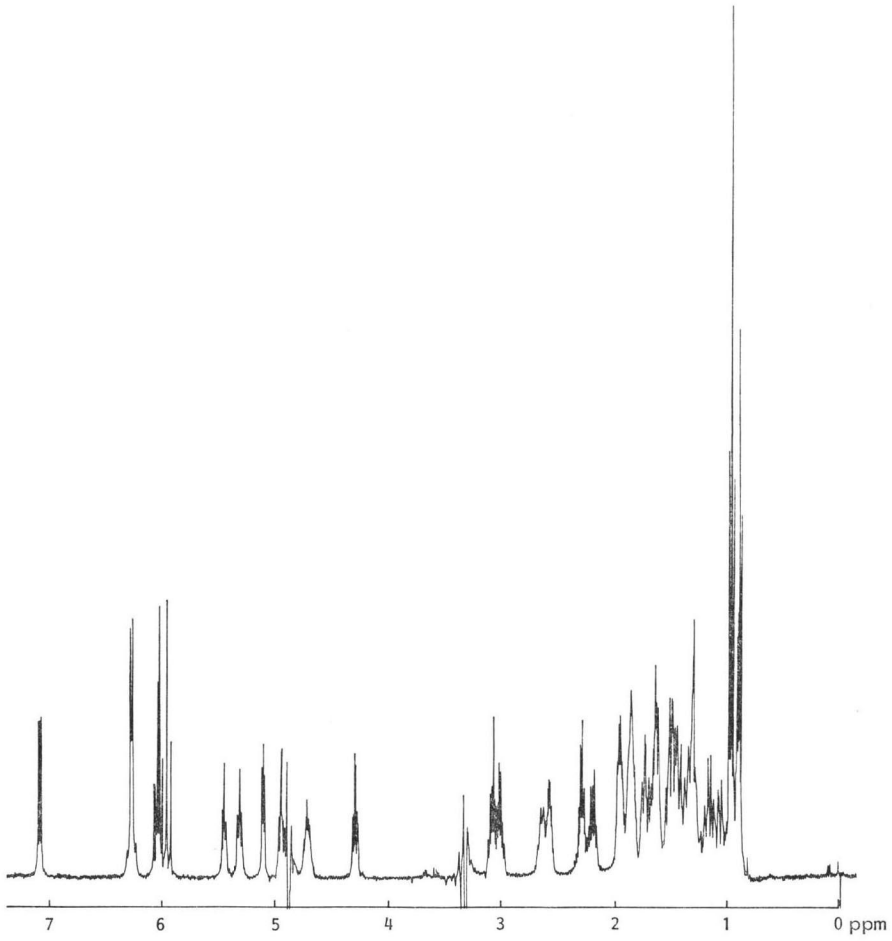


Fig. 4. ^{13}C NMR spectrum of phosphazomycin A (100 MHz, CD_3OD).

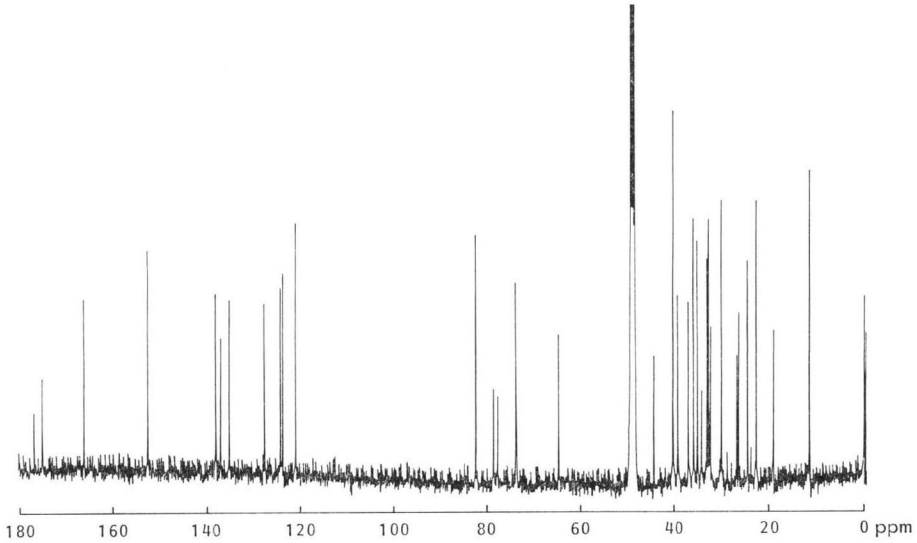


Table 1. Antimicrobial activity of phosphazomycin A.

Test organism	MIC (μg/ml)*
Aspergillus oryzae	0.9
Penicillium chrysogenum	0.22
Trichophyton mentagrophytes	0.45
Cochliobolus miyabeanus	0.11
Pyricularia oryzae	0.03
Rhizoctonia solani	7.5
Colletotrichum lagenarium	0.015
Botrytis cinerea	0.008
Glomerella cingulata	0.22
Alternaria mali	0.015
Fusarium oxysporum	30.0
Saccharomyces cerevisiae	15.0
Candida albicans	30.0

* Conventional agar dilution method was employed.

komamycins A and B[1] resemble phosphazomycin A in UV absorption. However, because they are described to contain several amino acids, they should be different from phosphazomycin A, which has no amino acid.

Phosphazomycin A showed strong growth inhibitory activity against various fungi and yeasts, but no activity against bacteria (Table 1). The inhibition of fungal growth by phosphazomycin A was accompanied by swelling of mycelia. It inhibited β-1,3-glucan synthetase from *Saccharomyces cerevisiae*[2] at the ID_{50} value of 100 μg/ml. However, the much lower MIC values (Table 1) need further study regarding the primary target of the antibiotic. In pot tests, the antibiotic showed a preventive value of 97% against cucumber gray mold disease and 88% against cucumber anthracnose when administered at the concentration of 25 ppm. Acute toxicity of the antibiotic was at a dose level of 19 mg/kg (LD_{50}, by oral administration to mice).

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