STUDIES ON NEW PHOSPHATE ESTER ANTIFUNGAL ANTIBIOTICS PHOSLACTOMYCINS

I. TAXONOMY, FERMENTATION, PURIFICATION AND BIOLOGICAL ACTIVITIES

SUSUMU FUSHIMI, SHIGERU NISHIKAWA†, AKIRA SHIMAZU and HARUO SETO*

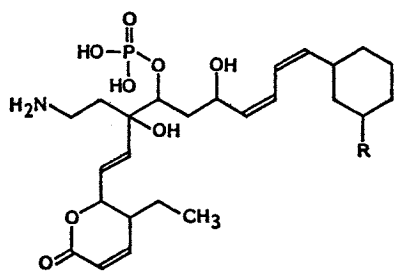
Institute of Applied Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
†Pesticides Research Laboratory, Takarazuka Research Center, Sumitomo Chemical Co., Ltd., Takatsukasa, Takarazuka, Hyogo 665, Japan

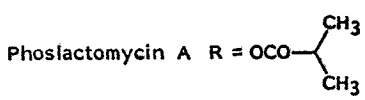
(Received for publication February 16, 1989)

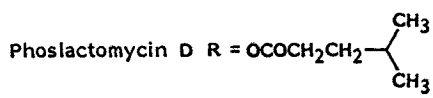
New antibiotics phoslactomycins A, B, C, D, E and F, which contain α,β-unsaturated δ-lactone, phosphate ester, conjugated diene and cyclohexane ring moieties, were isolated from the culture broth of a soil isolate actinomycete. Morphological, cultural and physiological studies revealed that the isolate is a strain of *Streptomyces nigrescens*. Phoslactomycins were obtained by butanol extraction, gel filtration and reverse phase chromatography. The antibiotics show strong activity against various fungi, particularly phytopathogenic fungi (*Botrytis cinerea* and *Alternaria kikuchiana*).

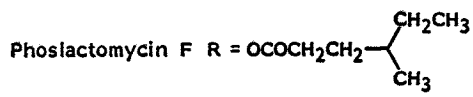
Gray mold disease caused by the infection with *Botrytis cinerea* is known as one of the several limiting factors for vegetable production. Although benzimidazole fungicides and polyoxins are being used agriculturally to control the disease, their effect has become insufficient due to the appearance of resistant strains. As a result of a study seeking new antibiotics active against this pathogen,

Fig. 1. Structures of phoslactomycins A to F.









we found that a soil isolate SC-273 collected at Kasai, Hyogo Prefecture, produced six new antibiotics named phoslactomycins A, B, C, D, E and F (Fig. 1). This paper deals with the taxonomy of the producing organism, fermentation, purification and biological activities of phoslactomycin. Physicochemical properties and structure elucidation of phoslactomycin will be described in the accompanying paper[1].

Materials and Methods

Screening Procedures

Soil isolate actinomycetes were inoculated in Erlenmeyer flasks (200 ml) containing 60 ml of the screening medium consisting of soluble starch 3.0%, yeast extract 1.0%, NaCl 0.3% and $CaCO_3$ 0.3% (pH 7.2) and were cultured for 4 days at 28°C on a rotary shaker (150 rpm). Broth filtrates were sprayed onto leaves of 2-week old cucumbers. After treatment with the broth filtrates, inoculation was made by putting nutrient agar containing conidia of *B. cinerea* onto the leaves. Inoculated plants were kept at 18°C for 3 days under high humidity and darkness. Antifungal activity was evaluated through a visual observation of the infected area.

Taxonomy

Taxonomy of the producing organism was performed by the method of SHIRLING and GOTTLIEB[2]. All cultures were incubated at 28°C and were observed for 14~21 days in the experiment for cultural characteristics. Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[3]. The results were obtained after 14 days at 28°C. The type of diaminopimelic acid in the cell wall was determined by the method of BECKER *et al.*[4].

Culture Conditions for *Streptomyces nigrescens* SC-273

Erlenmeyer flasks (500 ml) containing 100 ml of the same medium used for screening were inoculated with spores from a slant culture of SC-273 and incubated for 2 days at 28°C on a rotary shaker (150 rpm). The culture broth (600 ml) was transferred into a 50-liter jar fermenter with 30 liters of the production medium consisting of soybean meal 1.5%, dried yeast 0.2%, soluble starch 2.5% and $CaCO_3$ 0.4%, pH 6.2.

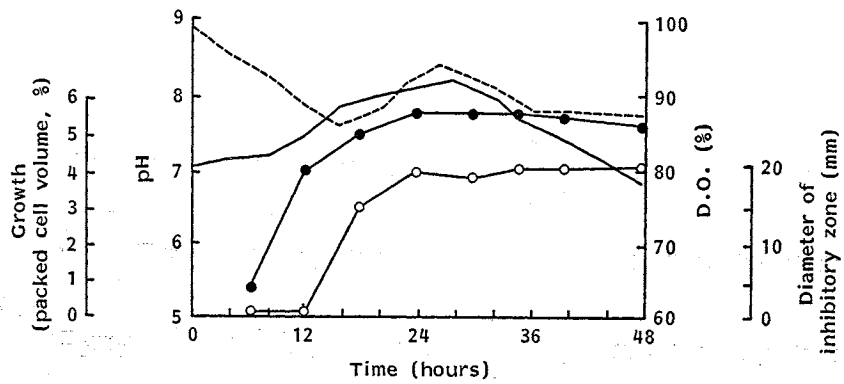
Fermentation was carried out for 2 days at 28°C under aeration of 30 liters/minute and agitation of 400 rpm. A typical time course is shown in Fig. 2. Production of phoslactomycin was assayed by the paper disc method using *Cladosporium fulvum* as the test microorganism. This fungus is suitable for the paper disc method due to the easily distinguishable green color of the mycelium.

Biological Activities of Phoslactomycin

MICs of phoslactomycin E (main product) were determined by the agar dilution method. Com-

Fig. 2. Time course of phoslactomycin production.

● Growth, ○ diameter of inhibitory zone, — pH, --- D.O.



parison of antimicrobial activity between phoslactomycins A, B, C, D, E and F was made by the paper disc method. Each paper disc contained 0.5 μg of the test compound.

Test media used for bacteria, yeast and fungi were nutrient agar, yeast extract - peptone - glucose agar and potato - glucose agar, respectively. The concentration of the tested microorganisms was 10^6 (spores or cells)/ml.

Results and Discussion

Taxonomy

Microscopic studies showed that aerial mycelia were formed from the branched substrate mycelia grown in various agar media. Matured spore chains were spiral and consisted of more than 10 spores per chain. Most of the spores were oval ($0.30 \sim 0.33 \times 0.65 \sim 0.70$ μm) and possessed a smooth surface (Fig. 3). Typical verticillate aerial mycelium and other special morphology were not observed. The cultural characteristics of strain SC-273 on various media are presented in Table 1. Physiological properties and utilization of carbon sources of strain SC-273 are summarized in Tables 2 and 3, respectively. Since the whole cell hydrolysate contained LL-diaminopimelic acid, the cell wall type of strain SC-273 was classified as Type I. The microbial characteristics of strain SC-273 were in good agreement with those of *S. nigrescens*. Therefore, the strain SC-273 was identified as *S. nigrescens*. This organism has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession No. of FERM P-9319.

Purification

The flow diagram of the purification for phoslactomycin is shown in Scheme 1. Since the activity was present in both culture filtrate and mycelia, phoslactomycin was extracted from both fractions after separation by continuous centrifugation. The broth filtrate (120 liters) was adsorbed on a column of Diaion HP-20. After the column was washed with water and 40% aq MeOH, successively, the antibiotic mixture was eluted with 50% aq acetone. The active eluate was combined with an acetone extract of the mycelial cake and then concentrated *in vacuo* to afford an aqueous solution which was extracted with butanol. The solvent layer was concentrated *in vacuo* to dryness. The oily residue (235 g) was dissolved in MeOH and chromatographed on a column of Sephadex LH-20 developed with MeOH. Active fractions were collected and concentrated to dryness under reduced pressure. The residue (25 g) was dissolved in 90% aq MeOH and subjected to Lobar column RP-8 (Merck) chromatography using 90% aq MeOH as an eluting solvent. Active fractions were combined and concentrated *in vacuo* to dryness. The residue (8.1 g) was dissolved in MeOH and chromatographed on a column of Toyopearl HW-40 F with MeOH. The active eluate was collected and concentrated *in vacuo* to afford a crude syrup. Further puri-

Fig. 3. Scanning electron micrograph of the spores of strain SC-273 (yeast extract - malt extract agar). The bar represents 0.5 μm.

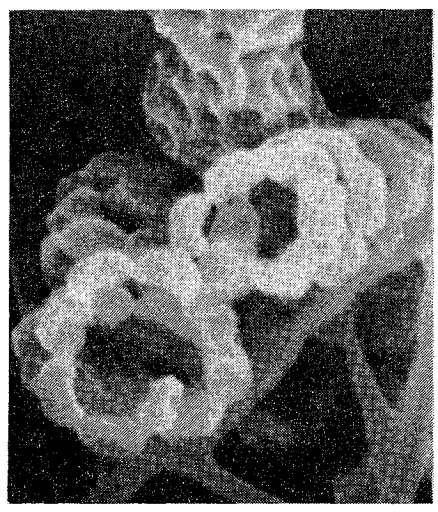


Table 1. Cultural characteristics of strain SC-273.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose - nitrate agar	Good	Moderate, white (a)	Pale yellowish brown (3gc~3ie)	Trace of brownish white (3ca)
Glucose - asparagine agar	Moderate	None	Pale yellowish brown (2gc~2ic)	None
Glycerol - asparagine agar (ISP-5)	Poor	None	Pale yellow (2ca)	None
Inorganic salts - starch agar (ISP-4)	Good	Good, white (a), gray (7ih~5ih)	Pale yellowish brown (3ie), brownish gray (4ig~4li)	Trace of pale yellow (2ca)
Tyrosine agar (ISP-7)	Moderate	None	Light brownish gray (2ec)	Trace of light brownish gray (4ec)
Nutrient agar	Moderate	None	Pale yellow (2ca)	None
Yeast extract - malt extract agar (ISP-2)	Good	Moderate, white (a), gray (2dc)	Pale yellowish brown (3ie), yellowish brown (3ng)	Trace of pale yellowish brown (3le)
Oatmeal agar (ISP-3)	Good	Poor, gray (3fe)	Light brownish gray (3ec)	Trace of pale yellowish brown (2gc)

Incubated at 28°C for 14 days.

Color and number in parenthesis followed "Color Harmony Manual", 4th Ed., Container Corporation of America, Chicago, U.S.A., 1958.

Table 2. Physiological properties of strain SC-273.

Property		Medium
Temperature range for growth	6.5~34.5°C	Yeast extract - malt extract agar
Optimum temperature range for growth	15~30°C	Yeast extract - malt extract agar
Gelatin liquefaction	Negative	Glucose - peptone - gelatin
Starch hydrolysis	Positive	Inorganic salts - starch agar
Milk coagulation	Negative	Skim milk
Milk peptonization	Positive	Skim milk
Reduction of nitrate	Negative	Bacto nitrate broth
Melanin formation	Negative	Tyrosine agar, peptone - yeast extract - iron agar, Tryptone - yeast extract broth

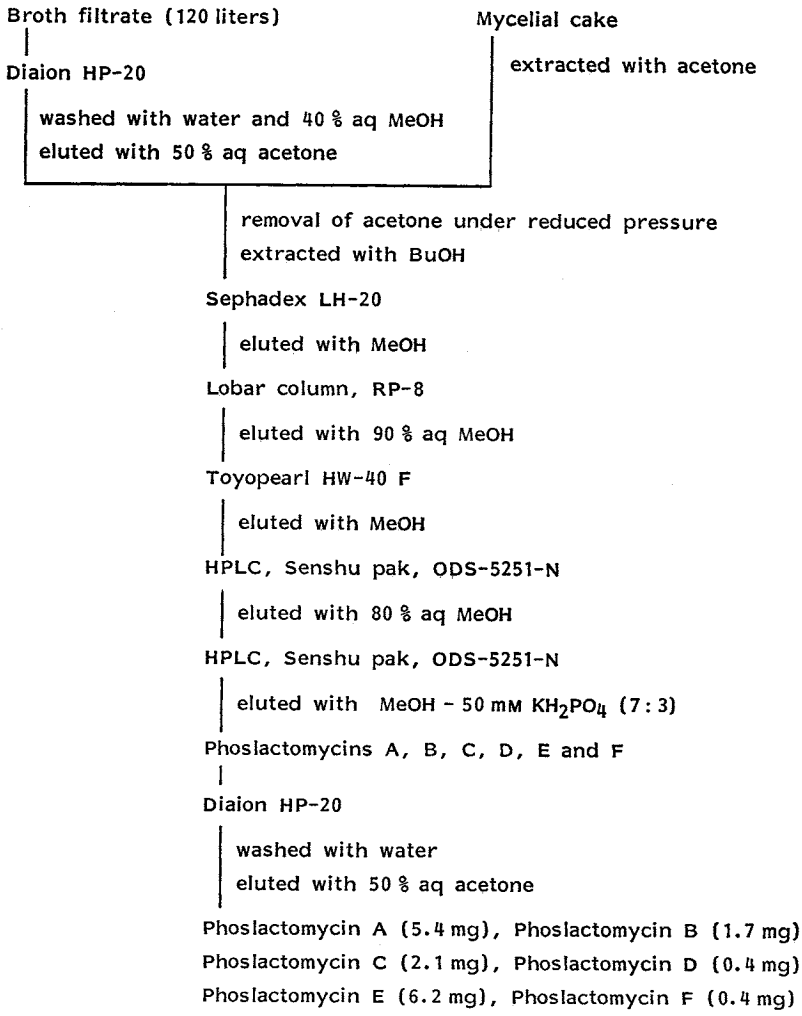
Table 3. Utilization of carbon sources by strain SC-273.

Response	Carbon source
Positive	D-Glucose, D-xylose, D-fructose, D-mannitol, sucrose, raffinose, inositol
Negative	L-Rhamnose, L-arabinose

The basal medium used was ISP-9.

fication was carried out using reverse phase preparative HPLC. The crude syrup was dissolved in 80% aq MeOH and subjected to reverse phase preparative HPLC using 80% aq MeOH as an eluting solvent. The active eluate was concentrated to give a pale yellowish powder. The crude powder was

Scheme 1. Purification procedure for phoslactomycins.



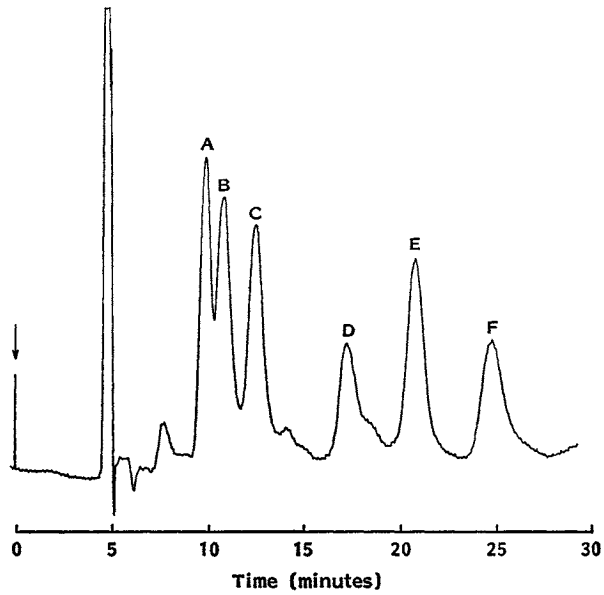
dissolved in 70% aq MeOH and subjected to reverse phase preparative HPLC with a solvent system consisting of MeOH - 50 mM KH_2PO_4 (7 : 3). In this preparative HPLC, the activity was detected in six fractions named A, B, C, D, E and F in the order of retention time. Each fraction was passed through a column of Diaion HP-20. After washing with water, each component of phoslactomycins was eluted with 50% aq acetone. After evaporation of the eluate, phoslactomycins A (5.4 mg), B (1.7 mg), C (2.1 mg), D (0.4 mg), E (6.2 mg) and F (0.4 mg) were obtained as colorless powders. The HPLC profile of phoslactomycin is shown in Fig. 4.

Physico-chemical properties and structure elucidation of phoslactomycins A to F are published in the accompanying paper[1].

Biological Activities

The antibacterial and antifungal spectra of phoslactomycin E (main product) are shown in Tables 4 and 5, respectively. The antibacterial activity of phoslactomycin E was very weak; it showed weak inhibitory effect against some Gram-positive bacteria.

Fig. 4. HPLC profile of the phoslactomycin mixture.



Column conditions: Column; Senshu pak ODS-H-1251, 4.6 i.d. × 250 mm, solvent; MeOH - 50 mM KH_2PO_4 (7 : 3), flow rate; 0.4 ml/minute, temperature; 25°C, detector; UV (233 nm).

Table 4. Antibacterial activity of phoslactomycin E.

Test organisms	MIC (μg/ml)
Corynebacterium glutamicum	>50.0
C. michiganense	18.8
Bacillus subtilis	>50.0
B. polymyxa	25.0
B. megaterium	18.8
Streptococcus faecalis	18.8
Agrobacterium tumefaciens	>50.0
Pseudomonas lachrymans	>50.0
P. glumae	>50.0
P. fluorescens	>50.0
Xanthomonas campestris	>50.0
Escherichia coli	>50.0
Erwinia carotovora	>50.0

Table 5. Antifungal activity of phoslactomycin E.

Test organisms	MIC (μg/ml)
Alternaria kikuchiana	<0.31
A. mali	<0.31
A. solani	<0.31
Rhizopus nigricans	1.25
Mycosphaerella melonis	<0.31
Fusarium oxysporum f. sp. *lycopersici*	3.13
Ustilago nuda	1.25
Aspergillus niger	1.25
Botrytis cinerea	<0.31
Chaetomium globosum	<0.31
Penicillium luteum	5.00
Verticillium albo-atrum	<0.31
Pseudocercosporella herpotrichoides	<0.31
Saccharomyces cerevisiae	20.00
Candida tropicalis	>20.00

Phoslactomycin E showed high antifungal activity, especially against *B. cinerea*, *Alternaria kikuchiana*, *Alternaria mali*, *Alternaria solani*, *Mycosphaerella melonis*, *Chaetomium globosum*, *Verticillium albo-atrum* and *Pseudocercosporella herpotrichoides*.

Comparison of antimicrobial activity between phoslactomycins A, B, C, D, E and F is shown in Table 6. Each compound had almost the same antimicrobial spectrum. Since the difference in the chemical structures between each compound is ascribed to a substituent bound to a cyclohexane ring, the substituent is considered not to be important for antimicrobial activity.

Since the chemical structures of phoslactomycins are quite different from those of benzimidazole

Table 6. Comparison of the antimicrobial activity among phoslactomycins A, B, C, D, E and F.

Test organisms	Diameter of inhibition zone (mm)					
	A	B	C	D	E	F
Cladosporium fulvum	23.5	22.0	25.0	27.0	28.0	25.0
Ustilago maydis	15.0	14.0	15.0	15.0	15.0	13.0
Pyricularia oryzae	13.0	13.0	12.0	11.5	11.5	11.5
Candida albicans	0	0	0	0	0	0
Escherichia coli	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	0	0

Paper disc method was used. Each paper disc contained 0.5 μg of phoslactomycin.

fungicides and polyoxins which are being used agriculturally, phoslactomycin is expected to have antifungal activity against resistant strains. The details of the biological activities of phoslactomycin will be published elsewhere.

Acknowledgments

We express our thanks to Miss M. TERAMOTO, Sumitomo Chemical Co., Ltd., for determining antimicrobial activities. We also thank to Drs. M. UEDA, K. KAMOSHITA, T. KATO and S. INOUE, Sumitomo Chemical Co., Ltd., for their encouragement throughout this work.

References

1) FUSHIMI, S.; K. FURIHATA & H. SETO: Studies on new phosphate ester antifungal antibiotics phoslactomycins. II. Structure elucidation of phoslactomycins A to F. J. Antibiotics 42: 1026~1036, 1989
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
4) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965