FOSFONOCHLORIN†, A NEW ANTIBIOTIC WITH SPHEROPLAST FORMING ACTIVITY

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A new antibiotic, fosfonochlorin, was found in the culture filtrate of four strains of fungi freshly isolated from soil samples. These strains were identified as *Fusarium avenaceum*, *Fusarium oxysporum*, *Fusarium tricinctum* and *Talaromyces flavus*.

Fosfonochlorin was a low molecular weight antibiotic (MW 158), soluble in water and methanol, but insoluble in acetone, ethyl acetate and chloroform. It was named after its possession of phosphorus and chlorine atoms, each one molar in its structure. The structure was determined as chloroacetylphosphonic acid mainly by the ^1H NMR and mass spectrometric analyses.

It was moderately active against some species of Gram-negative bacteria and its synergistic effect with glucose-6-phosphate was observed on *Staphylococcus aureus* and *Escherichia coli*.

Spheroplast formation of the susceptible organisms with this antibiotic suggested that it might inhibit their cell wall synthesis.

In the course of our screening program for new antibiotics with spheroplast formation, four strains of fungi identified as *Fusarium avenaceum*, *Fusarium oxysporum*, *Fusarium tricinctum* and *Talaromyces flavus* were found to produce a new identical antibiotic, named fosfonochlorin. The present paper deals with identification of the producing organisms, fermentation using *T. flavus*, isolation, biological and physico-chemical properties and structural elucidation of fosfonochlorin.

Identification of Producing Organisms

Strain SANK 15680 was freshly isolated from a soil sample collected at Taga-cho, Inugami-gun, Shiga Prefecture, Japan.

From the characters described below, the fungus was identified as *T. flavus*[1,2].

T. flavus (Klöcker) Stolk et Samson

Anamorphic state: *Penicillium dangeardii* Pitt

Colonies on CYA[1] medium grow rapidly, attaining a diameter of 33~35 mm in 7 days at 25°C, and are floccose to velutinous, grayish yellow (4-B-6)[3] in color. Reverse of the colonies is grayish orange (5-B-3). Gymnothecia develop abundantly but conidiogenesis is sparse. Pale yellowish

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soluble pigment is produced.

Colonies on MEA medium are similar in appearance to those on CYA medium, but gymnothecia develop more abundantly. Soluble pigment is not produced.

Colonies on G25N medium grow restrictedly, with conidiogenesis, attaining a diameter of 4~5 mm, but gymnothecia are not produced.

At 5°C on CYA medium germination of spores and conidia does not occur.

Colonies on CYA medium grow rapidly, attaining a diameter of 32~33 mm at 37°C. Gymnothecia and conidia develop very restrictedly. Soluble pigment is not produced. Reverse of the colonies are grayish orange (5-B-3) to grayish yellow (4-B-6).

Gymnothecia are composed of loosely entwined bright yellowish hyphae, globose, non-ostiolate, 200~500 μm in diameter. Initials of the gymnothecia consist of club-shaped ascogonia, up to 200 μm in length and 3~5 μm in diameter, around which thin antheridia coil tightly several times.

Asci are 8-spored, evanescent, subglobose, 8~13 μm in diameter. Ascospores are yellowish, ellipsoidal and with spines, 3.5~5.5×2.5~3.0 μm in size.

Conidiophores arise from the substratum stipes, which are 20~70×1.5~2.0 μm, smooth-walled. Penicilli are monoverticillate to biverticillate; metulae appressed, in verticils of 2~3, and are 10~15×2.0~2.3 μm; phialides are 2~5 per metula, acerose, 7.5~15×2.0~2.5 μm; conidia are ellipsoidal, 2.0~4.0×1.5~2.5 μm with smooth wall, born in short chains.

Strain SANK 10182 was freshly isolated from a soil sample collected at Tazawa-ko, Kitasenboku-gun, Akita Prefecture, Japan.

From the characters described below, the fungus was identified as *F. oxysporum*[4].

F. oxysporum Schlecht

Colonies on potato-sucrose agar grow rapidly, attaining a diameter of 45~50 mm in 4 days at 26°C. They are floccose with vinaceous mycelium, with a sterile whitish margin. Reverse of the colonies is yellowish brown. At 37°C, growth is nil.

Phialides of macroconidia are 10~30×3~5 μm. Macroconidia are hyaline, phialidic, fusoid-falcate, with a somewhat hooked apex and a foot cell, 2 to 6 transverse septa when mature. They measure: 2 septate 20~28×4.5~5.5 μm; 3 septate 25~35×4.5~5.0 μm; 4 septate 25~40×4.5~5.5 μm; 5 septate 35~50×4.5~5.5 μm; 6 septate 40~50×4.5~5.5 μm.

Phialides of microconidia are simple, 5~30×2.5~4.5 μm, Microconidia are hyaline, one-celled, oblong and somewhat curved, 5~10×3~5 μm.

Chlamydospores formed abundantly, both smooth and rough walled, subglobose, 8~12 μm in diameter, mostly terminal, generally solitary but occasionally formed in pairs. No teleomorph was observed.

Strain SANK 10282 was isolated from seed of persimmon picked at Fuchu-city, Tokyo, Japan.

Although teleomorph has not been observed, from the characters described below, the fungus was identified as *F. avenaceum*[4].

F. avenaceum (Fr) Sacc

Colonies on potato-sucrose agar grow rapidly, attaining a diameter of 50 mm in 4 days at 26°C. They are floccose, pale reddish purple. Reverse of the colonies is reddish purple. At 37°C growth is nil.

Conidiophores of macroconidia are of both polyblastic and phialidic types. Macroconidia are

hyaline, long fusoid and falcate, with a somewhat hooked apex and a foot cell, 3 to 5, rare 6, transverse septate when mature. They measure: 3 septate 28~45×3.5~4.5 μm; 4 septate 40~50×4.0~5.0 μm; 5 septate 45~63×4.0~5.0 μm.

Chlamidospores were not observed. Teleomorph has not been observed.

Strain SANK 10382 was isolated from the calyx of persimmon picked at Fuchu-city, Tokyo, Japan. From the characters described below, the fungus was identified as *F. tricinctum*.

F. tricinctum (Corda) Sacc

Colonies on potato-sucrose agar grow well, attaining a diameter of 40 mm in 4 days at 26°C. They are floccose, pale purpulish red at central part, nearly white at the margin. Reverse of the colonies is reddish purple. At 37°C growth is nil.

Conidiophores of macroconidia are phialidic, 10~30×3.0~5.0 μm. Macroconidia are hyaline, falcate, 2 to 5 transversely septate, but most of them are 3 to 4 septate. They measure: 2 septate 15~25×2.5~4.5 μm; 3 septate 20~25×2.5~4.5 μm; 4 septate 26~35×3.0~4.5 μm; 5 septate 26~35×2.5~4.5 μm.

Phialides of microconidia are simple, 5~20×2.5~4.5 μm in size. Microconidia are subglobose to elliptical, smooth-walled, 7.5~15.0×5.0~7.5 μm. No chlamydospore nor teleomorph was observed.

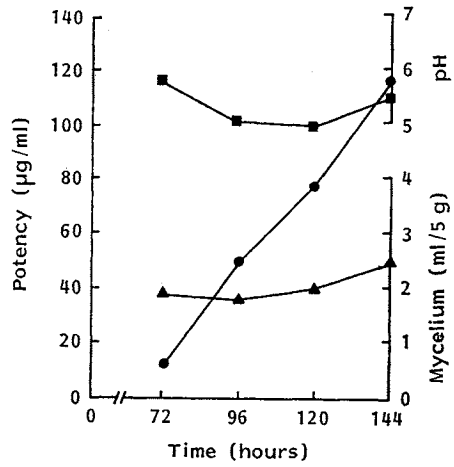
Production of Fosfonochlorin

One loopful growth of *T. flavus* SANK 15680 on potato - glucose agar was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of the medium composed of mashed potato 2.5%, sucrose 2.0%, Casamino acids 1.0%, KH_2PO_4 0.5% and $MgSO_4 \cdot 7H_2O$ 0.25%. The flasks thus inoculated were incubated on a rotary shaker (220 rpm) at 24°C for 96 hours. A 4-ml aliquot of the culture was seeded to each 500-ml Erlenmeyer flask containing 80 ml of the same medium described above and the fermentation in the 150 flasks was carried out at 24°C for 144 hours. The time course of fermentation is shown in Fig. 1.

The antibiotic production was monitored by conventional paper-disc agar diffusion assay using *P. mirabilis* as the test organism.

Fig. 1. Time course of fermentation of fosfonochlorin.

▲ Growth, ● potency, ■ pH.



Isolation

Approximately 10 liters of the culture filtrate were passed through an activated carbon column (1 liter) and the effluent was applied on a 600-ml of Dowex 21K column (CH_3COO^-). The antibiotic was eluted with 2.5% acetic acid - pyridine buffer at pH 5.0. The active fractions were pooled and concentrated to dryness under reduced pressure. The residue was solubilized with 100 ml of deionized water and the solution was applied on a column of Dowex 50W-X4 (H^+, 1 liter) and developed with water. The active fraction was further chromatographed on a

Fig. 2. Isolation and purification of fosfonochlorin.

Culture filtrate (10 liters)
↓ treated with activated carbon column
Effluent
↓
Dowex 21K (AcO^-, 600 ml)
↓ eluted with 2.5% AcOH - pyridine buffer at pH 5.0
Dowex 50W-X4 (H^+, 1 liter)
↓ developed with H_2O
Amberlite CG-50 (H^+, 1 liter)
↓ developed with H_2O
Silica gel prepn TLC PrOH - H_2O (7 : 3)
↓ eluted with 80% aq MeOH
Dowex 50W-X4 (H^+, 1 liter)
↓ developed with H_2O
neutralized with dil NaOH
lyophilized
Fosfonochlorin sodium salt (140 mg)

column of Amberlite CG-50 (H^+). These cation exchange column chromatographies were carried out effectively to remove a large amount of inorganic acids, especially phosphoric acid which was one of major constituents in the culture medium. Partially purified fosfonochlorin thus obtained was finally purified by preparative TLC on silica gel plates (No. 5715, Kieselgel 60 F254 Merck Co., Ltd.) developed with 70% propanol. The active eluate was passed through 1 liter of Dowex 50W-X4 (H^+) and developed with water. The active fraction was adjusted to pH 7.0 with diluted NaOH and lyophilized. Finally 140 mg of fosfonochlorin sodium salt was obtained as an amorphous, hygroscopic white powder.

Biological Properties

The MIC of fosfonochlorin against bacteria was determined by a serial 2-fold agar dilution method. The medium used was nutrient agar (Difco Co., Ltd.) and the MICs were determined after incubation at 37°C for 24 hours.

As shown in Table 1, fosfonochlorin was active against *P. mirabilis* and *P. vulgaris* and weakly active against *Salmonella enteritidis*, *Klebsiella pneumoniae* and *Providencia rettgeri*, but inactive against other bacteria tested even at a concentration of 200 μg/ml.

It was well known that fosfomycin (phosphonomycin)[5], related to fosfonochlorin in the structure and ability of spheroplast formation in the Gram-negative bacteria, was augmented its antimicrobial activities by combination with glucose-6-phosphate (G-6-P)[6]. We, therefore, tested reinforcement of the antibiotic with G-6-P. The presence of 50 μg/ml of G-6-P showed broadened antimicrobial spectrum of fosfonochlorin against Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp. and some species of *Shigella* and *Salmonella*.

Mice tolerated intravenous dose of 400 mg/kg of fosfonochlorin without any toxic syndrome.

Physical and Chemical Properties

Fosfonochlorin was obtained as sodium salt and the appearance was hygroscopic, white powder with $[\alpha]_D^{25}$ 0° (*c* 0.56, H_2O). The molecular formula of free form was established to be $C_2H_4O_4PCl$, with the molecular weight of 158 based on the analyses of field desorption mass spectra (FD-MS)

Table 1. Antimicrobial spectrum of fosfonochlorin with glucose-6-phosphate.

Test organism	MIC (μg/ml)	
	D-Glucose-6-phosphate	
	0 μg/ml	50 μg/ml
Staphylococcus aureus FDA 209P JC-1	>200	50
Enterococcus faecalis S-299	>200	25
Bacillus subtilis PCI 219	>200	>200
Micrococcus luteus PCI 1001	>200	>200
Escherichia coli NIHJ JC-2	>200	>200
E. coli B No. 5	>200	6.25
E. coli SANK 72875	>200	6.25
Klebsiella pneumoniae PCI 602	>200	>200
K. pneumoniae SANK 74975	200	6.25
Pseudomonas aeruginosa NCTC 10490	>200	200
Enterobacter cloacae SANK 70163	>200	200
Shigella flexneri 2a	>200	3.13
Salmonella enteritidis	25	3.13
Serratia marcescens SANK 73060	>200	100
Proteus vulgaris OX 19	6.25	1.56
P. mirabilis SANK 70461	1.56	1.56
P. mirabilis SANK 71873	1.56	3.13
Providencia rettgeri SANK 73775	100~200	200

Medium: Nutrient agar (Difco).
Inoculum size: 10^6 cells/ml.

Table 2. Physical and chemical properties of fosfonochlorin.

Nature	Acidic, white powder
$[\alpha]_D^{25}$	0° (*c* 0.56, H_2O)
Molecular formula	$C_2H_4O_4PCl$ (TMS derivative $C_2HO_4PCl \cdot 3TMS$ M^+ *m/z* 374)
Molecular weight	158 (FD-MS $(M+H)^+$ *m/z* 159)
Solubility	
Soluble:	H_2O, MeOH, DMF
Insoluble:	Acetone, EtOAc, $CHCl_3$
Rf (Merck No. 5715)	0.46 (PrOH - H_2O, 7 : 3)
Color reaction	BCG, I_2, 1% aq ammonium molybdate - 50% aq H_2SO_4 (+)
UV nm ($E_{1cm}^{1\%}$)	276 (19.7) in H_2O

($(M+H)^+$ *m/z* 159) and high-resolution electron impact mass spectra (HREI-MS) of trimethylsilyl derivative (M^+, found 374.0729, calcd for $C_{11}H_{28}O_4PClSi_3$ 374.0721).

It is soluble in water, methanol and dimethylformamide, but insoluble in acetone, ethyl acetate and chloroform. It gave positive reactions to bromocresol green, ammonium molybdate and sulfuric acid on silica gel plates.

The UV spectrum showed a maximum at 276 nm ($E_{1cm}^{1\%}$ 19.7) in acidic or neutral aqueous solution and 330 nm ($E_{1cm}^{1\%}$ 15.7) in 0.01 N sodium hydroxide solution. The IR spectrum of the antibiotic showed characteristic bands at 3400, 1700, 1600, 1400, 1200~1050 and 900 cm^{-1}.

In the 90 MHz ^1H NMR spectrum in D_2O, no detectable signal was observed except for a doublet at 3.6 ppm coupled with phosphorus (J_{H-P}=5.0 Hz).

The Rf value on a silica gel plate (No. 5715) was 0.46 when developed with a mixture of PrOH - H_2O, 7:3.

Structural Elucidation

Mass spectrum of the trimethylsilyl (TMS) derivative revealed a molecular ion at *m/z* 374 which comprised three TMS groups based on the 27 mass unit shift of the molecular ion in the spectrum of nonadeuterium-TMS derivative (Fig. 3). The fragment ion at *m/z* 225 was assigned as $PO(OTMS)_2$ and the base ion at *m/z* 298 was ascribed to the ion of $P(OTMS)_3$ which was probably given from the ion at *m/z* 225 by rearrangement in the ion chamber. Subtraction of the partial structure, $PO(OTMS)_2$ from the molecular formula gave us $C_2HOCl(TMS)$ (Fig. 3). Three

Fig. 3. Mass spectrum of TMS-derivative of fosfonochlorin.

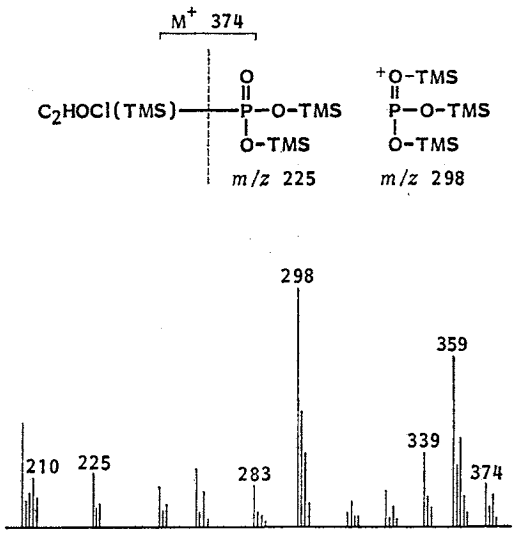


Fig. 4. Three possible structure of fosfonochlorin.

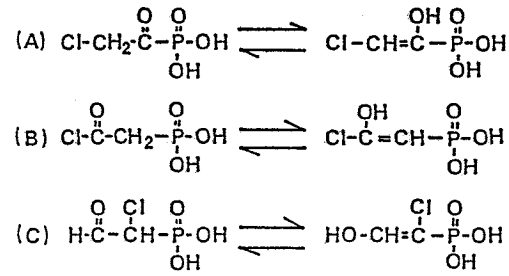


Fig. 5. ^{13}C NMR of fosfonochlorin in DMSO (A), DMSO+D_2O (B), H_2O (C).

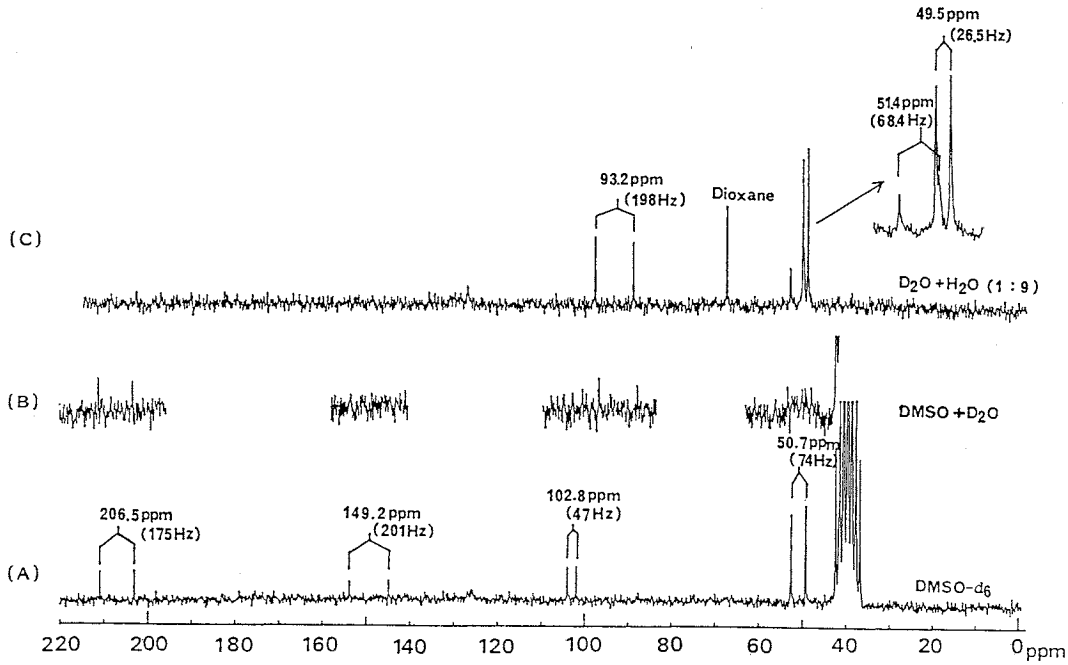
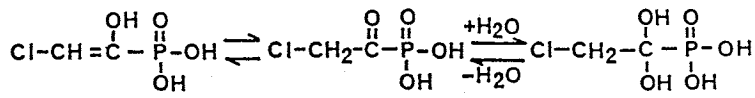


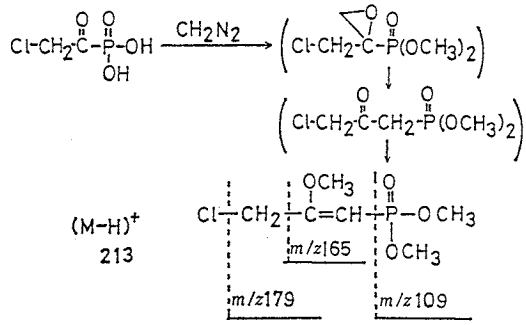
Fig. 6. Tautomeric system of fosfonochlorin.



possible structures, A, B and C, based on these considerations were shown in Fig. 4.

However, from the facts that instability of acid chloride in the aqueous solution and absence of carbonyl carbon signal due to aldehyde group in ^{13}C NMR, the possibilities of structures B and C were excluded and the most possible structure was assumed to be A. This structure A was further confirmed by ^{13}C NMR spectrometry shown in Fig. 5.

Fig. 7. Reaction with diazomethane.



The ^{13}C NMR spectrum of the antibiotic in DMSO-d_6 demonstrated four sets of signals (each of them coupled with phosphorus) for two-carbon compound and this phenomenon is ascribed to keto-enol isomerism of the compound (Fig. 5A). The peaks were assigned as follows: A methylene in keto-form at 50.7 ppm (J_{C-P}=74 Hz), a methine in enol-form at 102.8 ppm (J_{C-P}=47 Hz), an enol carbon at 149.2 ppm (J_{C-P}=201 Hz) and a ketone carbon at 206.5 ppm (J_{C-P}=175 Hz). The coupling constants with phosphorus of these carbons were well agreed with the values cited in the literature. To get the evidences of this keto-enol relationship, a few drops of D_2O were added to NMR tube expecting that methylene and methine signals would disappear by the exchange reaction. As shown in Fig. 5B, the signals at 50.7 ppm and 102.8 ppm due to methylene and methine carbons, disappered and a ketone signal remained. The enol peak at 149.2 ppm, however, also disappeared and a signal at 93.2 ppm with one bond C-P coupling (J_{C-P}=198 Hz) was newly appeared. This finding could be explained by the formation of hydrate based on the chemical shift of each resonance. This fact was further confirmed by the ^{13}C NMR spectrum in H_2O (10% D_2O was added for NMR lock) as shown in Fig. 5C, in which the signals due to hydrate were dominated.

In summary, the antibiotic exists in three forms, *i.e.* keto, enol and hydrate, depending on the solvent (Fig. 6).

The treatment of the antibiotic with diazomethane, only resulted in rearranged product shown in Fig. 7. This 1, 3 dipolar addition also supports the structure of fosfonochlorin since SEKINE[7] have reported the same type of reaction with dechlorinated analogue of the antibiotic. The evidences and the arguments described above firmly established the structure of fosfonochlorin as structure A.

These all spectral data indicated the structure of fosfonochlorin afforded in the equilibrium with enol and keto form in DMSO and further with keto and hydrate form in aqueous solution.

Discussion

The antibiotic produced by several species of fungi was characterized with a unique structure having chlorine and phosphorus in the molecule (MW 158). Several antibiotics with C-P bond in the struc-

ture such as fosfomycin, bialaphos (SF-1293)[8], phosphinothricin[9] phosalacine[10], FR-900098, FR-32863, FR-33289, FR-31564[11] and fosfazinomycins A and B[12] have been reported, but this is the first report of the production of such antibiotic by fungi. The mode of action of fosfonochlorin is supposed to be the inhibition of the cell wall synthesis because of its spheroplast forming activity in *P. mirabilis* and its inhibition of incorporation of [^{14}C]diaminopimelic acid into the acid insoluble fraction of *E. coli* H2143 (unpublished data). It is feasible to think that fosfonochlorin would act as an analogue of phosphoenolpyruvate (PEP) in the PEP-GlcNAc-3-*O*-enolpyruvate transferase reaction which is the target of fosfomycin. Fosfonochlorin would mimic PEP probably as an enol form in the enzyme reaction and alkylate the enzyme by the very active α-halo ketone function.

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