Communications to the Editor

ISOLATION AND STRUCTURE OF PHOSPHAZOMYCIN C

Sir:

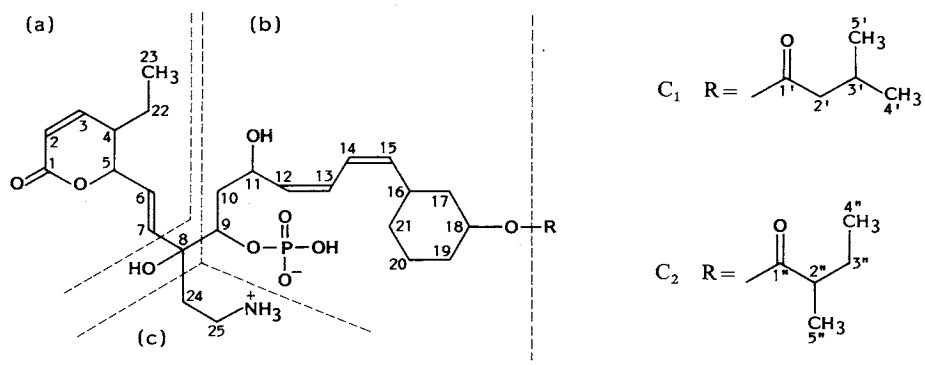
In 1985, we reported[1] the isolation of phosphazomycin A, one of the components of an antifungal antibiotic complex produced by *Streptomyces* sp. HK-803.[2] Recently, we have succeeded in the isolation of component C. Although purified phosphazomycin C gave a single peak in HPLC analysis, NMR spectroscopic evidence showed that it is a mixture of two components, C_1 and C_2. Here we wish to report the isolation and structure elucidation of the phosphazomycin C complex (Fig. 1).

The strain was cultured at 28°C for 90 hours in a tank fermenter containing 600 liters of a production medium composed of corn starch 5.0%, glucose 1.5%, soybean flour 2.5%, beer yeast 0.4%, meat extract 0.1%, and NaCl 0.2%. The culture broth (560 liters) was filtered, and the mycelial cake was extracted with 70% aqueous acetone. After removal of the acetone, it was combined with the broth filtrate and passed through a column of Diaion HP-20. After washing with 40% aqueous methanol, the antibiotics were eluted with 50% aqueous acetone. Acetone was removed by evaporation and the resulting solution was extracted with butanol. The organic layer was concentrated to dryness. The residue was dissolved in a small volume of methanol and applied onto a column of activated charcoal, which was then eluted with 50% aqueous acetone. Active fractions were collected, concentrated *in vacuo*, and lyophilized to afford a crude powder of the phosphazomycin complex (30 g). This was dissolved in a small amount of methanol and subjected to chromatography on a column of MCI-GEL (CHP-20P) packed with 50% aqueous acetone and developed with the same solvent. Fractions containing phosphazomycin C were collected, concentrated *in vacuo* and lyophilized. For further purification, the powder was subjected to Sephadex LH-20 chromatography with 50% aqueous acetone. Crystalline material was precipitated from the concentrated solution of fractions rich in phosphazomycin C. After filtration, 30 mg of phosphazomycin C were obtained as a colorless crystalline powder. HPLC analysis showed a single peak (Sensyu pak ODS-N, CH_3CN: 1% triethylamine - phosphoric acid (pH 7), 80 : 20).

The complex melts at 191 ~ 193°C with decomposition. It is optically active; $[\alpha]_D^{21}$ +81° (*c* 1.0, MeOH). Negative and positive FAB-MS gave *m/z* 612 $(M-H)^-$ and *m/z* 614 $(M+H)^+$, respectively. Elemental analysis; Calcd for $C_{30}H_{48}O_{10}NP \cdot H_2O$: C 57.05, H 7.92, N 2.22, Found: C 56.82, H 7.78, N 2.22. The UV spectrum showed an absorption maximum at 233 nm (ε 46,800) in MeOH. The IR spectrum (KBr tablet) exhibited absorption peaks at 3400, 3275, 2975, 1725, 1630, 1460, 1385, 1250, 1185 and 1055 cm^{-1}. Phosphazomycin C is soluble in methanol, ethanol and alkaline water, slightly soluble in acetone and water, and insoluble in chloroform, benzene, and ether. It gave positive reactions to ninhydrin, Lemieux and anisaldehyde-sulfuric acid reagents. The antibiotic has strong antifungal activity. The MICs are shown in Table 1.

In the ^{31}P spectrum, a signal was observed at δ_P

Fig. 1. Structures of phosphazomycins C_1 and C_2.



3.30 ppm (d, $J_{P-O-C-H}=9.2$ Hz) suggesting the presence of phosphate ester moiety. A ^1H NMR spectrum of phosphazomycin C is shown in Fig. 2. Chemical shifts from the ^1H and ^{13}C NMR spectra with peak assignments are listed in Tables 2 and 3, respectively. Analyses of ^1H-^1H and ^1H-^{13}C COSY spectra afforded partial structures (a), (b), and (c) as shown in Fig. 1. Bond linkage from C-1 to C-7 including an ethyl side chain, C-22–C-23, and from C-9 to C-16 including a phosphate substituent at C-9 were deduced and supported with ^1H-^{31}P COSY spectrum. ^1H signals of methylenes (17-H, 19-H, 20-H, 21-H, 24-H and 25-H) were complex and could not be assigned completely. However, decoupling experiments revealed the presence of a cyclohexyl group and an isolated CH_2CH_2 group. On treatment of phosphazomycin C with acetic anhydride in pyridine, a diacetyl derivative was formed. In the ^1H NMR spectrum, down field shifts were observed for the three protons of 11-H and 25-H (11-H, 0.85 ppm; 25-H, 0.12 and 0.34 ppm). Positive ninhydrin reaction and the chemical shifts of 25-H suggested the presence of an amino group on C-25, which was supported by the downfield shift just noted after acetylation. The configurations of the four double bonds were deduced from ^1H-^1H coupling constants (Δ^2Z, $J=9.8$ Hz; Δ^6E, $J=16$ Hz; Δ^{12}Z, $J=9.8$ Hz; Δ^{14}Z, $J=10.0$ Hz). The partial structures (a), (b), and (c) plus one tertiary carbon bearing oxygen equal 25 carbon atoms. In the ^{13}C NMR spectrum, an additional 10 carbon signals with relatively low intensity were observed. By analyses of ^1H-^{13}C and ^1H-^1H COSY spectra and decoupling experiments, *iso*- and *anteiso*-valeroyl substituents were deduced. The intensities of the corresponding proton signals (2′-H–5′-H, 2″-H–5″-H) were approximately one half of the other proton signals. These results indicate that phosphazomycin C is a mixture containing two isomeric acyl groups in an approximately equal molar ratio. The positions of the acyl groups were determined by long range selective proton decoupling (LSPD) experiment between 18-H and carbonyl carbons. α,β-Unsaturated-δ-lactone was supported by a cross peak between C-1 and 5-H. The connectivity of C-7, C-9 and C-24 through C-8 was deduced by observation

Table 1. Antifungal activity of phosphazomycin C.

Test organism	MIC (μg/ml)[a]
Aspergillus oryzae IFO 5239	1.6
A. niger	1.6
Penicillium chrysogenum	0.4
Trichophyton mentagrophytes IFO 6202	0.4
Cochliobolus miyabeanus IFO 5277	1.6
Pyricularia oryzae IFO 5994	0.8
Rhizoctonia solani IFO 6258	25
Colletotrichum lagenarium IFO 7513	0.05
Botryotinia fuckeliana IFO 5365	0.0004
Glomerella cingulata IFO 9767	0.2
Alternaria mali IFO 8984	0.025
Fusarium oxysporum IFO 9761	12.5

[a] Conventional agar dilution method was employed using potato-sucrose medium. MICs were observed after 72 hours incubation.

Fig. 2. ^1H NMR spectrum of phosphazomycin C (500 MHz, CD_3OD).

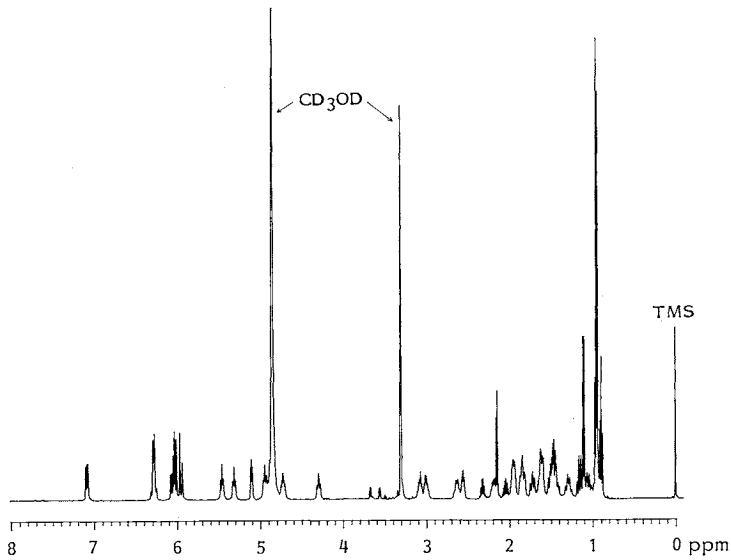


Table 2. ^1H NMR spectrum in CD_3OD of phosphazomycin C with assignment.

Proton No.	δ_H, ppm (J in Hz)	Proton No.	δ_H, ppm (J in Hz)
2-H	6.01 d (J=9.8)	19-H_2	1.29 m, 1.96 m
3-H	7.08 q (J=7.0, 9.8)	20-H_2	1.43 m, 1.84 m
4-H	2.56 m	21-H_2	1.05 m, 1.60 m
5-H	5.12 dd (J=6.0, 7.0)	22-H_2	1.48 m, 1.63 m
6-H	6.06 dd (J=7.0, 16.0)	23-H_3	0.95 t (J=7.8)
7-H	5.95 d (J=16.0)	24-H_2	1.96 m, 2.19 m
9-H	4.30 t (J=9.2)	25-H_2	3.00 m, 3.08 m
10-H_2	1.52 m, 1.72 m	2'-H_2	2.15 d (J=7.0)
11-H	4.95 br t (J=9.8)	3'-H	2.05 m
12-H	5.46 br t (J=9.8)	4'-H_3	0.94 d (J=7.0)
13-H	6.28[a]	5'-H_3	0.94 d (J=7.0)
14-H	6.29[a]	2''-H	2.32 m
15-H	5.31 br t (J=10.0)	3''-H_2	1.45 m, 1.59 m
16-H	2.63 m	4''-H_3	0.89 t (J=7.8)
17-H_2	1.16 m, 1.86 m	5''-H_3	1.10 d (J=7.8)
18-H	4.72 m		

[a] J value could not be measured.

Table 3. ^{13}C NMR spectrum in CD_3OD of phosphazomycin C with assignment.

Carbon No.	δ_C, ppm	Carbon No.	δ_C, ppm
C-1	166.4 s	C-19	32.5 t
C-2	121.2 d	C-20	24.5 t
C-3	152.7 d	C-21	33.1 t
C-4	40.6 d	C-22	22.7 t
C-5	82.4 d	C-23	11.4 q
C-6	127.7 d	C-24	34.5 t
C-7	137.5 d	C-25	37.2 t
C-8	77.8 s	C-1'	174.3 s
C-9	78.7 d	C-2'	44.7 t
C-10	40.6 t	C-3'	27.0 d
C-11	64.8 d	C-4'	22.7 q
C-12	135.4 d	C-5'	22.7 q
C-13	124.3 d	C-1''	178.0 s
C-14	123.8 d	C-2''	42.5 d
C-15	138.2 d	C-3''	28.0 t
C-16	36.2 d	C-4''	11.9 q
C-17	39.5 t	C-5''	17.1 q
C-18	74.0 d		

of cross peaks from 6-H, 7-H, 9-H, 24-H and 25-H to C-8.

Several similar antibiotics appeared recently, *i.e.*, antifungal substances from *Streptomyces hygroscopicus* MA.5000[3] and AF-273[4] from *Streptomyces nigrescens*. However, direct comparison of these substances with the phosphazomycins has not yet been achieved.

Acknowledgment

The authors wish to express their thanks to Dr. J. UZAWA of this institute for NMR measurement and Kaken Pharmaceutical Co., Ltd. for tank fermentation.

TETSUJI TOMIYA
MASAKAZU URAMOTO
KIYOSHI ISONO*

Antibiotics Laboratory,
RIKEN, The Institute of
Physical and Chemical Research,
Wako-shi, Saitama 351-01,
Japan
(Received April 26, 1989)

References

1) URAMOTO, M.; Y.-C. SHEN, N. TAKIZAWA, H. KUSAKABE & K. ISONO: A new antifungal antibiotic, phosphazomycin A. J. Antibiotics 38: 665~668, 1985
2) ISONO, K.; M. URAMOTO, G. NAKAMURA & H. KUSAKABE (RIKEN): A novel antibiotic HK-803 and its production method. Jpn. Kokai 31689 ('84), 1984
3) BURG, R. W.; M. HILL, L. J. COLE & S. HERNANDEZ (Merck): Antifungal substances and process for their production. U.S. 4575500, Mar. 11, 1986
4) FUSHIMI, S.; K. FURIHATA, S. NISHIKAWA, A. SHIMAZU & H. SETO: Studies on new antifungal antibiotics, AF-273, produced by Streptomycetes. Abstracts Papers of Annual Meeting of the Agricultural Chemical Society of Japan, No. 2IICp12, p. 214, Niigata, Apr. 2, 1989