STUDIES ON NEW PHOSPHONIC ACID ANTIBIOTICS

IV. STRUCTURE DETERMINATION OF FR-33289, FR-31564 AND FR-32863

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The structure of novel phosphonic acid antibiotics, FR-33289, FR-31564, and FR-32863, produced by strains of *Streptomyces*, have been established as **I**, **II**, and **III**, respectively, on the basis of spectroscopic and chemical evidences.

The excellent chemotherapeutic activity displayed by penicillins and cephalosporins prompted our interest in the search for inhibitors of bacterial cell wall biosynthesis, which led to the discovery of a novel phosphonic acid antibiotic, FR-900098[1].

A further screening program for new antibiotics revealed that other strains of *Streptomyces*[2] produced new three related compounds[3] containing phosphonic acid function.

In this paper, the structure elucidation of these antibiotics is described.

Structure of FR-33289 (I)

Antibiotic **I** was isolated as a white powder. Infrared absorption of **I** at 1630 cm^{-1} indicated the presence of an amide and absorption at 1150 cm^{-1} showed P=O function. Since the ultraviolet spectrum of **I** showed an end absorption, **I** has no aromatic ring.

The ^{1}H-nmr spectrum of **I** showed N-acetyl protons at δ 2.16, methylene protons at δ 1.88 (2H, dd, $J_{CH\text{-}CH_2}=6$ Hz, $J_{CH_2\text{-}P}=18$ Hz). Furthermore, this nmr spectrum showed one proton at δ 4.30 and two protons at δ 3.75, which were attributable to CH–O (or N) and CH_2–N (or O).

Positive color reaction to $FeCl_3$ reagent suggested that **I** contained a hydroxyamino function.

Table 1. Chemical structure.

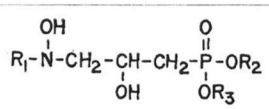
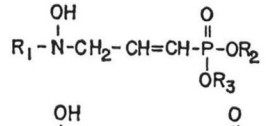
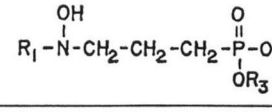
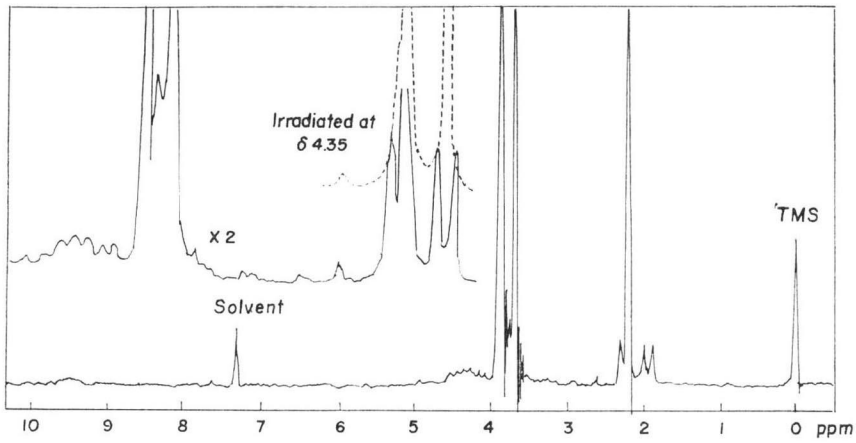
			R_1	R_2	R_3
FR-33289		**I**	CH_3CO	H	Na
		Ia	CH_3CO	CH_3	CH_3
		Ib	H	H	Na
FR-32863		**II**	CHO	H	K
		IIa	CHO	CH_3	CH_3
FR-31564 FR-900098		**III**	CHO	H	Na
		IV	CH_3CO	H	Na

Fig. 1. ^{1}H-Nmr spectrum of FR-33289 dimethyl ester ($CDCl_3$).



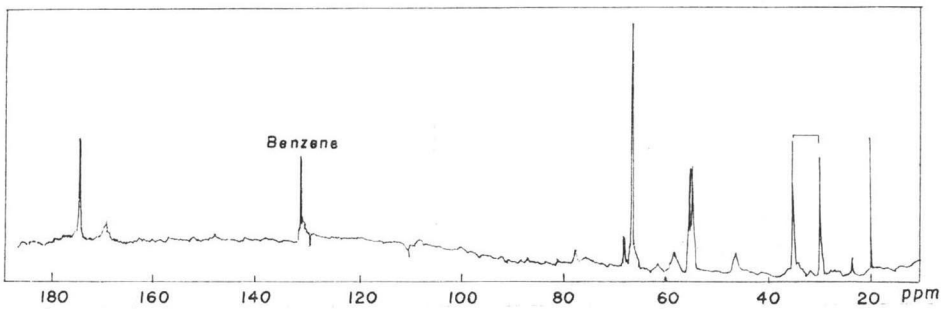
Phosphonic acid function was also suggested by positive color reaction to molybdate reagent, and paper electrophoresis. Phosphonic acid function was supported by the following spectral data; in the ^{1}H-nmr spectrum of **Ia**, the methyl signal appears as doublet ($J_{P\text{-}O\text{-}CH_3}=18$ Hz at 3.75). The proton decoupled ^{13}C-nmr spectrum of **I** showed a doublet (J=131 Hz) centered at δ 34.20 attributed to the C-1 carbon.

Treatment of the free acid of **I** with diazomethane gave dimethyl ester (**Ia**) (colorless oil), mass spectrum of which showed the molecular ion peak at *m/e* 241. Elemental analysis and mass spectrum of **Ia** established the molecular formula $C_7H_{16}NO_6P$.

Fig. 1 shows the ^{1}H-nmr spectrum of **Ia**. The spin decoupling experiment established that hydroxy function was attached on C-2. The double doublet at δ 2.17 (2H, J=6 Hz, 18 Hz) attributed to C-1 protons changed to doublet (2H, J=18 Hz) on irradiation of the one proton multiplet at δ 4.35. This indicated that CH–OH was adjacent to CH_2–P moiety. From chemical shifts and the coupling constants, the proton signals can be assigned as shown in Table 3.

Furthermore, ^{13}C-nmr spectrum in D_2O was in agreement with the above assignment. The proton decoupled spectrum of **I** is shown in Fig. 2. The signals due to C-1 and C-3 couple with phosphorus, (J=131 Hz and J=12 Hz, respectively), while the C-2 signal appears as singlet and the coupling ^{2}J (C-C-P) can not be observed. In the off-resonance spectrum, C-1 and C-3 appear as double triplets and C-2 appears as doublet. The assignments of these signals are summarized in Table 2.

Fig. 2. ^{13}C-Nmr spectrum of FR-33289 (D_2O).



Acid hydrolysis of **I** gave a product (**Ib**), which was positive to $FeCl_3$-potassium ferricyanide reagent. The ^1H-nmr spectrum revealed that **Ib** was 2-hydroxy-3-(N-hydroxy)aminopropylphosphonic acid. The optical rotation $[\alpha]_D^{25}$ was $+30°$ (c 0.2, H_2O).

The configuration of **Ib** was found to be R-configuration by comparison with the optical rotation of chemically synthesized compound.

The aforementioned results established that the structure of **I** is 3-(N-acetyl-N-hydroxy)-amino-2-hydroxypropylphosphonic acid. Finally, infrared spectrum, nmr spectra, antimicrobial activity and Rf value on TLC are consistent with those of chemically synthesized sample[4].

Table 2. Carbon-13 nmr data assigned to I.

δ ppm	Complete decoupling	Off-resonance	Assignment
20.31	s	q	CH_3CO-
31.59 36.81	d, J=131Hz	t of d	$-CH_2-P-$
54.77 55.25	d, J=12Hz	t of d	$-N-CH_2-$
65.44	s	d	-CH-OH
175.32	s	s	CH_3CO-

D_2O solution, δ in ppm relative to TMS using benzene as external standard.
Refer to structure **I** in Table 1.

Structure of FR-32863 (II)

Antibiotic **II** was isolated as colorless crystals. It was unstable in acidic solution. Color test (positive reaction to $FeCl_3$ and molybdate reagents), infrared spectrum and paper-electrophoresis suggested that **II** also contained phosphonic acid and hydroxyamino function in the molecule.

The ^1H-nmr spectrum of **II** showed a formyl proton at δ 8.02 and δ 8.38 (1H, two singlets), a methylene proton at δ 4.30 (2H, m) and two protons centered at δ 6.15 (2H, m), which are attributable to olefinic protons. The signals of the olefinic protons were overlapped together and gave no precise information because they lacked fine structure. On the other hand, ^1H-nmr spectrum of **IIa** showed satisfactory signals for the analysis of the two olefinic protons as shown in Fig. 3. The signals were observed at the separated chemical shifts each other. The decoupling experiment indicated the presence

Fig. 3. ^1H-Nmr spectrum of FR-32863 dimethyl ester (d_6-acetone).

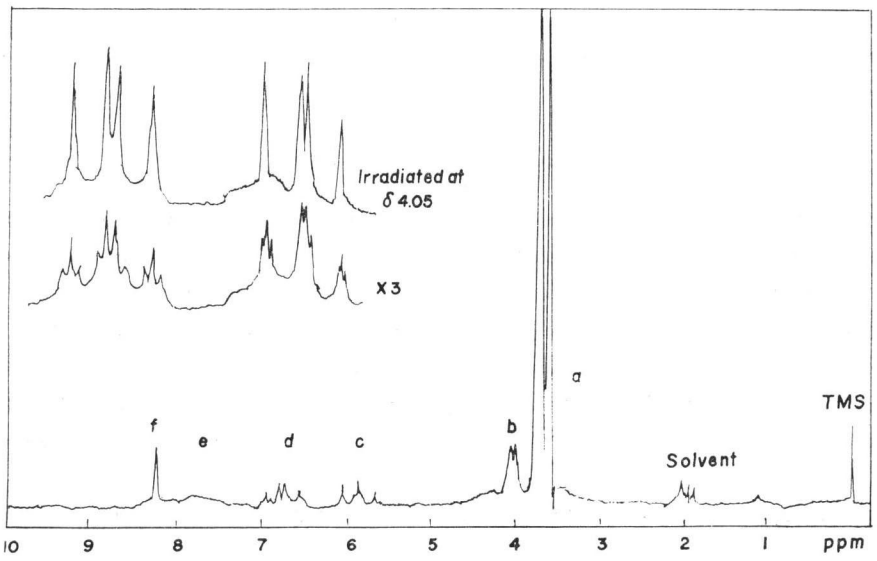
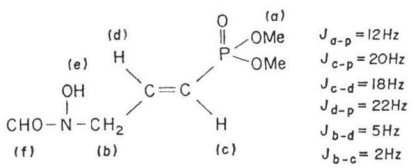


Fig. 4. Coupling constant of compound **IIa**.



of propene moiety. The multiplet signals both at δ 5.85 and δ 6.75 attributable to olefinic protons changed into two double doublets on irradiation of the multiplet signals at δ 4.05 assigned to C-3 methylene protons. The location of double bond on C-1 was decided from the fact that the couplings with phosphorus were observed in the two signals due to olefinic protons, but not in the signals due to methylene protons. The double bond of **II** was proved to be *trans*-configuration on the basis of the coupling constant ($J_{CH=CH}$ = 18 Hz). The assignments are summarized in Table 3 and Fig. 4.

Table 3. Proton magnetic resonance data assigned to **Ia**, **IIa**, **III**.

Compound	δ ppm	Area	Multiplicity[1]	Coupling (Hz)	Assignment
Ia[2]	2.17	2	d,d	J=6,18	$-CH_2-P$
	2.20	3	s		CH_3CO-
	3.70 } 3.80 }	6	d	J=18	$-P-O-CH_3$
	3.60~3.90	2	m		$-N-CH_2-CH-$
	4.35	1	m		$>CH-OH$
	9.50	1	broad s		-OH
IIa[3]	3.65 } 3.75 }	6	d	J=12	$-P-O-CH_3$
	4.05	2	m		$-N-CH_2-$
	5.85	1	d,d of t	J=2,18,20	$-CH=CH-P-$
	6.75	1	d,d of t	J=5,18,22	$-CH=CH-$
	7.80	1	broad s		-N-OH
	8.23	1	s		CHO-N-
III[4]	1.25~2.30	4	m		$-CH_2-CH_2-P$
	3.65	2	t	J=6	$-N-CH_2-$
	8.00 } 8.35 }	1	two s		CHO-N-

1) Multiplicity: m=multiplet, t=triplet, d=doublet, s=singlet
2) Spectrum obtained in $CDCl_3$, at 60 MHz. δ relative to TMS.
3) Spectrum obtained in d_6-acetone, δ relative to TMS.
4) Spectrum obtained in D_2O, δ relative to TSP.

Elemental analysis and mass spectrum of **IIa** established the molecular formula of $C_6H_{12}NO_5P$.

From above-mentioned results, it has been established that the chemical structure of **II** is 3-(N-formyl-N-hydroxy)amino-1-*trans*-propenylphosphonic acid.

Chemical and biological characteristics of FR-32863 have coincided with those of chemically synthesized authentic sample[4].

Structure of FR-31564 (III)

Antibiotic **III**, obtained as colorless crystals, was considered to possess phosphonic acid and hydroxyamino function from its color reactions (positive to $FeCl_3$ and molybdate tests) and infrared spectrum. Elemental analysis and titration established the molecular formula of $C_4H_9NO_5PNa$.

As shown in Fig. 2a, in the preceding paper, the ^{1}H-nmr spectrum of **III** showed that the signals at δ 1.25~2.30 (4H, m) and δ 3.65 (2H, t, J=6 Hz) are virtually identical with those observed in the ^{1}H-nmr spectrum of FR-900098 (**IV**), reported previously[1,3]. The two singlets at δ 8.00 and δ 8.35 (1H) were observed in the nmr spectrum of **IV**. The assignments of these signals are summarized in Table 3.

These data suggested that **III** has the structure of 3-(N-formyl-N-hydroxy)aminopropylphosphonic acid.

The elucidated chemical structure of FR-31564 has been corroborated by comparison of their infrared spectrum, nmr spectra and Rf value on TLC with those of chemically synthesized sample[5].

Experimental

Spectroscopic methods

^{1}H-Nmr spectra were recorded on a JEOL PS-100 spectrometer operating at 100 MHz unless otherwise mentioned. ^{13}C-Nmr was recorded on the same instrument operating in the FOURIER transform mode. Chemical shifts are reported as part per million (ppm) relative to tetramethylsilane, unless otherwise mentioned. Infrared and ultraviolet spectra were recorded by the use of HITACHI-215 spectrometer and HITACHI-323 spectrometer, respectively. Optical rotation was measured on a JASCO DIP-SL automatic polarimeter. Mass spectrum data were obtained using HITACHI RUM-6M mass spectrometer.

Melting points were measured on a THOMAS-HOOVER apparatus and are uncorrected.

Thin-layer chromatography

Thin-layer chromatography was carried out on Eastman chromatogram sheets. The spots for the natural products and their methyl esters were detected by spraying with 1% ferric chloride solution in ethanol and for hydroxamino derivative by further spraying with 1% potassium ferricyanide in water.

Paper electrophoresis

Paper electrophoresis was conducted at 300 volts for 2 hours with 0.2 M phosphate buffer pH 6.5. The object spots were detected by color test and/or bioautography.

Materials

Fermentation, isolation procedures, infrared spectra and nmr spectra were reported in the preceding paper[3].

FR-33289 (**I**)

$C_5H_{11}NO_6PNa$ (m.w. 235)

IR (Nujol): ν_{max} 3200, 2950, 2850, 2400, 1740, 1630 (–CO–N–), 1470, 1420, 1380, 1240, 1150 (–P=O), 1050, 965, 900, 740 cm^{-1}

^{1}H-nmr: δ 1.88 (2H, dd, J_{CH-CH_2}=6 Hz, J_{CH_2-P}=18 Hz), 2.16 (3H, s, CH_3–CO), 3.66~3.90 (2H, m, –N–CH_2–), 4.30 (1H, m, CH–OH)

FR-32863 (**II**)

$C_4H_7NO_5PK$ (m.w. 219) m.p. 176~180°C (decomp.)

IR (Nujol): ν_{max} 2960, 2870, 2600, 2350, 1665 (–CO–N–), 1530, 1460, 1440, 1400, 1380, 1365, 1290, 1250, 1180 (P=O), 1125, 1070, 1010, 980, 960, 950, 890, 830, 780, 700 cm^{-1}

^{1}H-nmr: δ 4.30 (2H, m, –N–CH_2–CH=), 6.0~6.4 (2H, m, –CH=CH–P), 8.02 (s) and 8.38 (s) (1H, CHO–N)

FR-31564 (**III**)

$C_4H_9NO_5PNa$ (m.w. 205) m.p. 189~191°C (decomp.)

IR (Nujol): ν_{max} 3600~2200, 1675 (–CO–N–), 1510, 1270, 1230, 1165 (P=O), 1015, 985, 920, 885 cm^{-1}

^{1}H-nmr (D_2O): δ 1.2~2.2 (4H, m, –CH_2–CH_2–P), 3.62 (2H, t, $J_{N-CH_2-CH_2}$=6 Hz), 8.00 (s) and 8.35 (s) (1H, CHO–N–)

Methylation of FR-33289 (I)

Methylation was performed as follows: to obtain the free acid of **I**, a solution of 200 mg of FR-33289 in 10 ml of water was applied to a column of CM-Sephadex (H^+ cycle, 10 ml). The effluent and wash were combined and 200 ml of methanol was added. To this solution, diazomethane in ether was added dropwise at 0°C until yellow color of diazomethane did not disappear. The solvent was evaporated under reduced pressure, and the residue was subjected to a column chromatography on silica gel with a mixture of chloroform - methanol (6: 1). The fractions containing the object compound were concentrated under reduced pressure to give 150 mg of FR-33289 dimethyl ester (**Ia**) as colorless oil.

$[\alpha]_D^{25}$ $-5.1°$ (c 0.75, MeOH)
m/e 241 (M^+)
IR (film): ν_{max} 3400, 2950, 2850, 1750, 1630 (–CO–N–), 1450, 1420, 1370, 1330, 1230, 1190 (P=O), 1095, 1030, 850, 830, 790, 770, 720 cm^{-1}
TLC: silica gel sheet (solvent: chloroform - methanol, 5: 1) Rf=0.45
Anal. Calcd. for $C_7H_{16}NO_6P$: C 34.85; H 6.64; N 5.81; P 12.86
Found: C 34.59; H 6.70; N 5.61; P 12.43

Acid hydrolysis of FR-33289

A solution of 1 g of FR-33289 in 18 ml of 1 N hydrochloric acid was allowed to stand for 1 hour at 80°C. The hydrolysate was purified by cellulose column chromatography using a solvent of 70% *n*-propanol. The positive fractions in color tests ($FeCl_3$-potassium ferricyanide) were collected and concentrated to give 100 mg of powder. This powder was dissolved in 25 ml of water and applied to Sephadex G-25 column (one liter) and was developed with water. Positive fractions in color tests were collected and lyophilized to yield 25 mg of white powder (**Ib**).

$[\alpha]_D^{25}$ $+30°$ (c 0.2, H_2O)
nmr (D_2O): δ 1.90 (2H, dd, J=6, 18 Hz), 2.80~3.70 (2H, m), 4.33 (1H, m)
TLC: cellulose sheet (60% aqueous propanol) Rf=0.53,
silica gel sheet (60% aqueous propanol) Rf=0.40
Anal: Calcd. for $C_3H_9NO_5PNa$: C 18.65; H 4.66; N 7.25
Found: C 18.60; H 4.71; N 7.10

Methylation of FR-32863 (**II**)

A solution of 100 mg of FR-32863 in 10 ml of water was applied to a column of CM-Sephadex (H^+ cycle, 10 ml) to obtain the free acid. The effluent and wash were combined and added with 200 ml of methanol. To this solution, diazomethane in ether was added dropwise at 0°C until yellow color of diazomethane did not disappear. The solvent was evaporated under reduced pressure. The residue was applied to a column of silica gel and the object compound was eluted with chloroform - methanol (19: 1). The fractions which were positive in ferric chloride test were collected and concentrated under reduced pressure to give a residual oil. This purification procedure was repeated once again to yield 50 mg of pure FR-32863 dimethyl ester (**IIa**) as a colorless oil.

m/e 209 (M^+)
IR (film): ν_{max} 3300, 3050, 2950, 2850, 1750, 1675, 1640, 1535, 1460, 1390, 1240, 1190 (P=O), 1050, 1030, 830 cm^{-1}
TLC: silica gel sheet (chloroform - methanol, 20: 1) Rf=0.1
Anal. Calcd. for $C_6H_{12}NO_5P$: C 34.45; H 5.74; N 6.70; P 14.83
Found: C 34.40; H 5.73; N 6.62; P 13.53

Discussion

Structure studies of our new antibiotics described in this report have characterized them as a series of phosphonic acids containing N-acylhydroxamino function. Several microbial products containing phosphonic acid have been reported[6~8]. Among them, fosfomycin is of considerable interest because of its inhibitory activity on the biosynthesis of bacterial cell wall. Our antibiotics also have an inhibitory effect on bacterial cell wall synthesis. But they are distinctly distinguished from fosfomycin by chemical and biological properties.

N-Formylhydroxamino function, found in our antibiotic, is rarely encountered in nature. So far, only hadacidin[9] has been known to possess N-formylhydroxamino function.

The structure elucidation and the determination of antibacterial activity of this group of antibiotics have given us some insight into their structure-activity relationship, which can be summarized as follows: (1) change in the propyl chain has only weak effect on the antibacterial activity, (2) substitution in the acyl moiety of these antibiotics has a profound effect on their efficacy; so far N-formyl gives the strongest activity.

Thus, these phosphonic acids seem to be promising starting material for a number of semisynthe-

tic antibiotics.

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