STRUCTURE DETERMINATIONS OF ENZYMATICALLY PHOSPHORYLATED PRODUCTS OF AMINOGLYCOSIDIC ANTIBIOTICS BY PROTON MAGNETIC RESONANCE

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The enzymatically inactivated products of kanamycin, paromamine and dihydrostreptomycin have been investigated by proton magnetic resonance at 100 MHz in deuterium oxide solution. The low-field shift of resonance position and the coupling constant of P-O-C-H support the previously proposed structures *viz* kanamycin-3′-phosphate, paromamine-3′-phosphate and dihydrostreptomycin-3′′-phosphate, respectively.

As reported in our previous paper[1~6], kanamycin and paromamine were inactivated in the presence of ATP and Mg^{++} by enzymatic preparations obtained from *Escherichia coli* K 12 carrying R factor or of *Pseudomonas aeruginosa* and the structures of the inactivated antibiotics were characterized as kanamycin-3′-phosphate and paromamine-3′-phosphate, respectively. Two types of streptomycin-inactivating enzymes are known in *E. coli* carrying R factor. One catalyses the reaction of streptomycin with ATP to yield streptomycin-3′′-adenylate[6,7] whereas the other produces streptomycin-3′′-phosphate.[8] Recently, MITSUHASHI *et al*[9]. also isolated dihydrostreptomycin-3′′-phosphate as the product formed from inactivation of dihydrostreptomycin by *P. aeruginosa*. The structures of these phosphorylated aminoglycosidic antibiotics were primarily deduced from periodate oxidation and hydrolysis studies.

In this paper, we report determinations of the structures of phosphorylated kanamycin, paromamine and dihydrostreptomycin by proton magnetic resonance studies. Since the structures of these compounds were successfully confirmed by the application of double resonance technique including the $^{1}H-\{^{1}H\}$ internuclear double resonance (INDOR) method[10], this technique utilizing a small amount of the material should be an important method in the determination of the structures of enzymatically inactivated antibiotics.

Materials and Methods

Inactivated antibiotics: Kanamycin-3′-phosphate was obtained, as previously described, from the inactivation of kanamycin with an enzyme preparation from *E. coli* K 12 ML 1629[3]. Paromamine-3′-phosphate was a product from enzymatic inactivation with an extract from *P. aeruginosa* NIHJ B-328[5]. Dihydrostreptomycin-3′′-phosphate[9] prepared by phosphorylation of dihydrostreptomycin with an enzyme

P. aeruginosa TI-13 was kindly supplied by Prof. SUSUMU MITSUHASHI, Gunma University.

Proton magnetic resonance (PMR): PMR spectra were obtained with a Varian HA-100 D spectrometer in deuterium oxide using tetramethylsilane as the external reference ($\delta=0$). The spectrometer modified by an INDOR kit (Nippon Electric Varian, Ltd.) was employed for the INDOR method. Concentrations of the samples were as follows: Kanamycin-3′-phosphate, 8.3 mg in 0.3 ml; paromamine-3′-phosphate (hydrochloride), 5.0 mg in 0.3 ml (pH 2); dihydrostreptomycin-3″-phosphate (sulfate), 5.9 mg in 0.3 ml (pH 4); kanamycin base, 12.3 mg in 0.3 ml; paromamine trihydrochloride, 5.0 mg in 0.3 ml; dihydrostreptomycin sulfate, 7.2 mg in 0.3 ml (pH 5).

Results and Discussion

The PMR spectrum of kanamycin showed two signals in the anomeric region (Fig. 1). Irradiation at δ 5.79 (J=3.3 Hz, 1′-H) caused complicated signals to collapse to a distinguishable doublet at δ 4.03 (J=9.5 Hz, 2′-H). It was confirmed by the irradiation at δ 4.03 that the chemical shift of the next proton (3′-H) existed in a slightly lower field than the 2′-H. The signal of 3′-H was shown to be located at approximately δ 4.25 by ^{1}H-{^{1}H} INDOR methods[10]. Irradiation at δ 5.50 (J=3.8 Hz, 1″-H) caused the complex signal to collapse to a clearly distinguishable doublet of J= 10.5 Hz at δ 3.96 (2″-H). Irradiation at δ 3.96 suggested that the chemical shift of 3″-H to high-field was due to the effect of amino group. These results indicate that the (1″-H)-(2″-H)-(3″-H) series of signals described above are attributable to the 3-aminoglucose moiety. Therefore, the signals of the (1′-H)-(2′-H)-(3′-H) series described above must belong to the 6-aminoglucose moiety.

In the PMR spectrum of kanamycin-3′-phosphate (Fig. 2), irradiation of 1′-H at δ 5.95 (J=3.5 Hz) caused the complex signals of 2′-H to collapse to a sharp doublet at the center of δ 4.17 (J=10.0 Hz). Irradiation at δ 4.17, attributed to 2′-H, caused

Fig. 1. PMR spectrum of kanamycin.

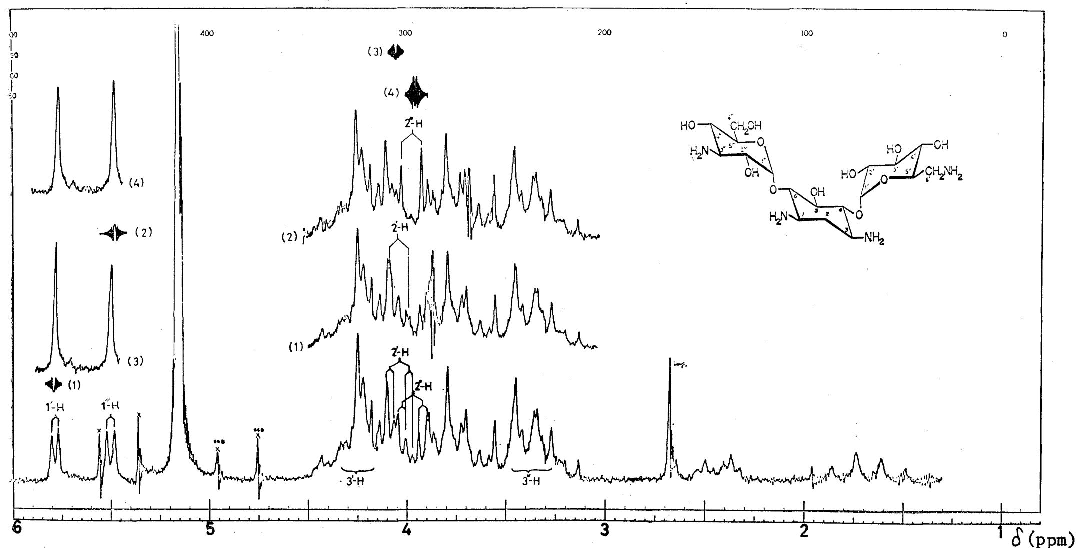
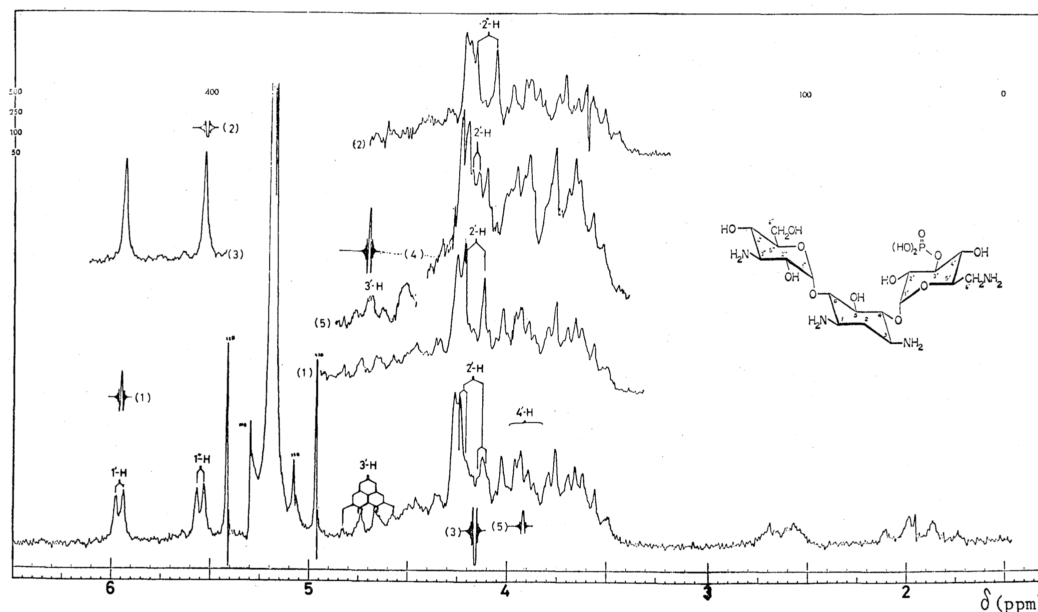


Fig. 2. PMR spectrum of kanamycin-3′-phosphate.



a quartet type signal at the center of δ 4.70 to collapse to a triplet type signal. Irradiation at δ 4.71 caused the signal of 2′-H to collapse to a doublet (J=3.5 Hz) and signals of 4′-H at δ 3.9 to change slightly. Irradiation at δ 3.90, attributed to 4′-H, caused a quartet signal of 3′-H to collapse to a triplet (J=*ca.* 8 Hz). In the spectrum of kanamycin-3′-phosphate, the resonance position of the 3′-H in 6-aminoglucose shifts by 0.45 ppm to a lower field than that of kanamycin (Table 1), and the splitting pattern shows the spin-spin coupling between P and 3′-H (J=8.0 Hz).

Table 1. Chemical shifts and coupling constants for the 6-aminoglucose moiety in kanamycin and kanamycin-3′-phosphate

	Chemical shift (δ)			Coupling constant (Hz)			
	1′-H	2′-H	3′-H	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{3',P}$
Kanamycin (base)	5.79	4.03	4.25	3.3	9.5	—	—
Kanamycin-3′-phosphate (base)	5.95	4.17	4.70	3.5	10.0	8.0	8.0

Table 2. Chemical shifts and coupling constants for the 2-aminoglucose moiety in paromamine and paromamine-3′-phosphate

	Chemical shift (δ)			Coupling constant (Hz)			
	1′-H	2′-H	3′-H	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{3',P}$
Paromamine (hydrochloride)	6.13	3.95	4.42	3.8	11.0	8.5	—
Paromamine-3′-phosphate (hydrochloride)	6.18	4.12	4.92	3.6	10.5	8.5	8.0
Paromamine-3′-phosphate (base)	5.95	3.84	4.77	3.5	10.0	8.0	8.0

In paromamine-3′-phosphate (Table 2), the low-field shift of 3′-H by 0.50 ppm and splitting caused by the phosphoryl group are also confirmed. The resonance position of 3′-H in the glucosamine moiety of paromamine was determined by the ^{1}H-{^{1}H} INDOR method. The INDOR responses (3′-H) obtained by monitor line 6 of 2′-H resonance are indicated in Fig. 3. Monitor line 3 produced responses in the same position but with the peaks inverted. Thus, the 3′-H proton was confirmed at δ 4.42 as a doublet of doublets (J=11.0, 8.5

Table 3. Chemical shifts and coupling constants for the N-methylglucosamine moiety in dihydrostreptomycin and dihydrostreptomycin-3″-phosphate

	Chemical shifts (δ)			Coupling constant (Hz)		
	1″-H	2″-H	3″-H	$J_{1'',2''}$	$J_{2'',3''}$	$J_{3'',4''}$
Dihydrostreptomycin (sulfate)	6.01	3.77	4.43	3.5	10.5	8.0
Dihydrostreptomycin-3″-phosphate (sulfate)	6.09	3.94	*ca.* 4.8	3.0	10.3	—
Dihydrostreptomycin-3″-phosphate (base)	5.93	3.49	*ca.* 4.8	3.0	10.2	—

Hz). In the case of paromamine-3′-phosphate, the 2′-H signal (δ 4.12, J=3.6, 10.5 Hz) was indicated by the irradiation of the anomeric proton 1′-H at δ 6.18 (J=3.6 Hz) (Fig. 4). Irradiation at δ 4.92 caused signals of 2′-H to collapse to doublet (J=3.6 Hz), and signals of 4′-H at the same position to simplify (J=9.5 Hz). Irradiation of the 2′-H and 4′-H at δ 4.14 caused the multiplet of 3′-H at δ 4.92 to reduce to a doublet ($J_{P-O-C-H}$=8.0 Hz) indicating that the 3′-H is located on a carbon bearing phosphoric ester function. Furthermore, irradiation of the signal of ^{31}P (40.49113 MHz) caused the signal of 3′-H at δ 4.92 to collapse to a doublet of doublets ($J_{2'3'}$=10.5 Hz and $J_{3',4'}$=8.5 Hz). These results indicate that the phosphoryl group in inactivated kanamycin or paromamine is attached equatorially at the C-3 positions in the respective 6-aminoglucose or 2-aminoglucose moieties.

The PMR spectra of dihydrostreptomycin sulfate and dihydrostreptomycin-3″-phosphate sulfate both showed two signals in the anomeric regions (Figs. 5 and 6). RINEHART *et al.*[11] assigned the δ 5.78 (J=1.6 Hz, d) signal of dihydrostreptomycin to the 1′-H of streptose, and we also confirmed that by the following experiments. Irradiation at δ 5.78 (1′-H) caused the doublet at 4.75 (2′-H, $J_{1',2'}$=1.6 Hz) to reduce to a sharp singlet. Irradiation of the signal at the center of δ 4.76 (4′-H, $J_{4',5'}$=6.5 Hz, q) overlapping with the signal at δ 4.75 (2′-H) produced a singlet at δ 1.68 ($J_{4',5'}$=6.5 Hz, d), methyl group of streptose, and the doublet of the anomeric proton of streptose collapsed to a singlet at δ 5.78. Thus, the signal for the anomeric proton

Fig. 3. PMR spectrum of paromamine trihydrochloride.

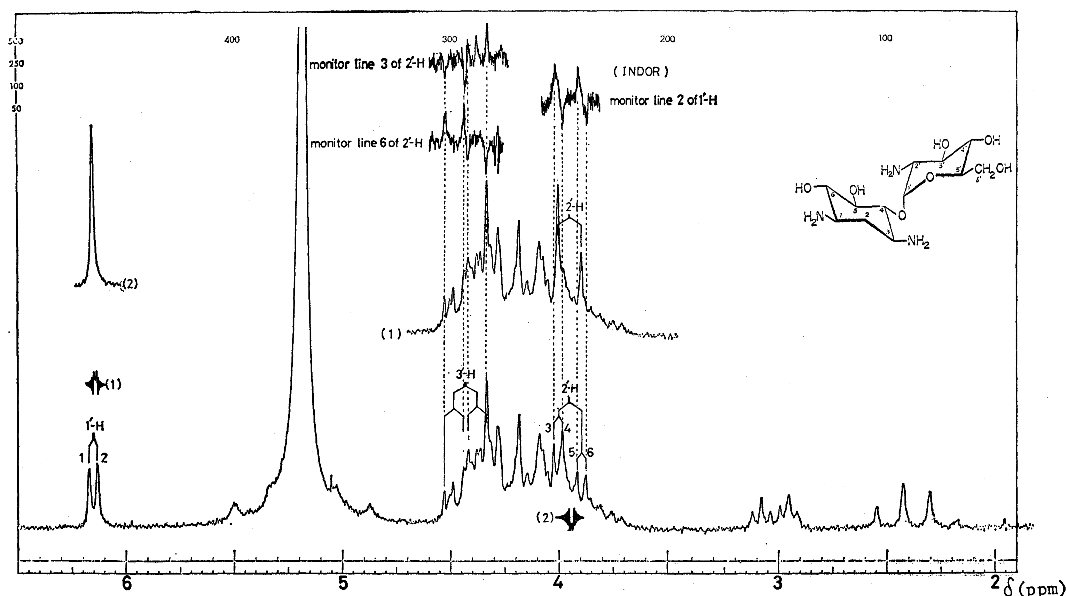
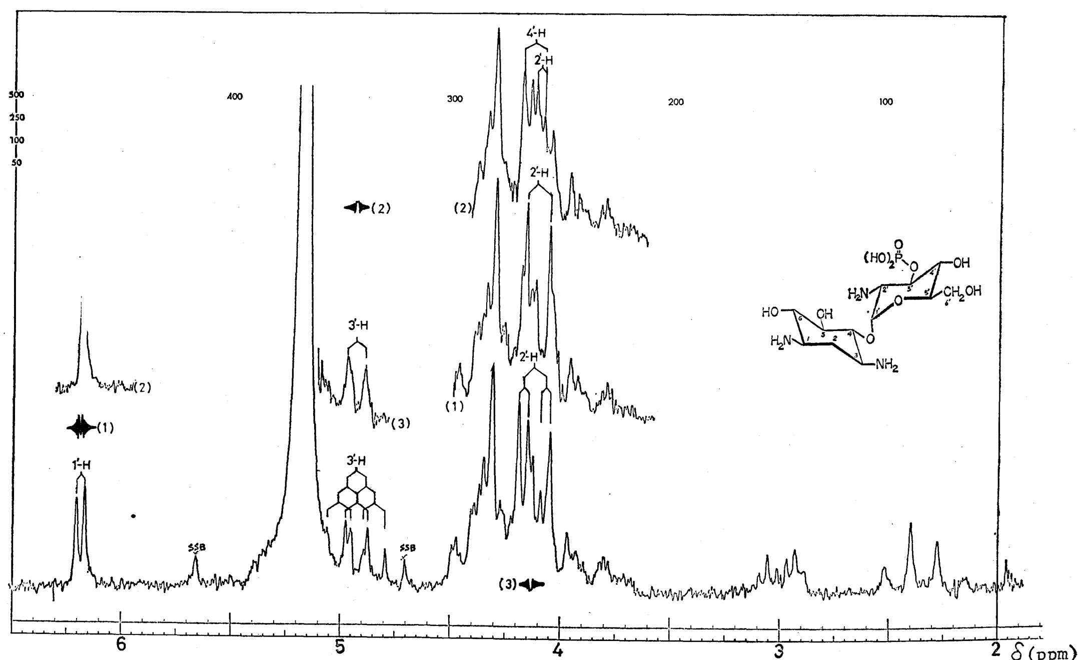


Fig. 4. PMR spectrum of paromamine-3′-phosphate (hydrochloride)



of N-methylglucosamine should be the signal at δ 6.01 (1″-H, J=3.5 Hz, d). Irradiation of the signal at δ 6.01 caused the doublet of doublets signal at δ 3.77 (2″-H, J=10.5, 3.6 Hz) to reduce to a doublet (J=10.5 Hz). Irradiation at δ 3.77 caused the doublet of doublets signal at δ 4.43 (3″-H, J=10.5, 8.0 Hz) to reduce to a doublet (3″-H, J=8.0 Hz) and a doublet signal at δ 6.01 (J=3.5 Hz) to collapse to the singlet signal (1″-H). Accordingly, the signals at δ 6.01, 3.77 and 4.43 can be assigned to 1″-H, 2″-H and 3″-H of N-methylglucosamine, respectively. In the case of dihydro-

Fig. 5. PMR sprectrum of dihydrostreptomycin sulfate.

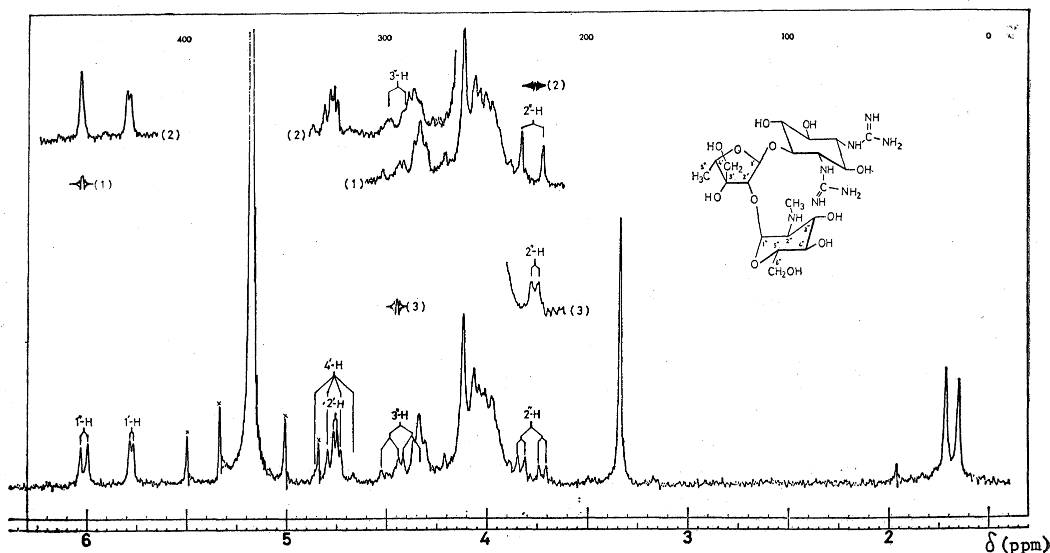
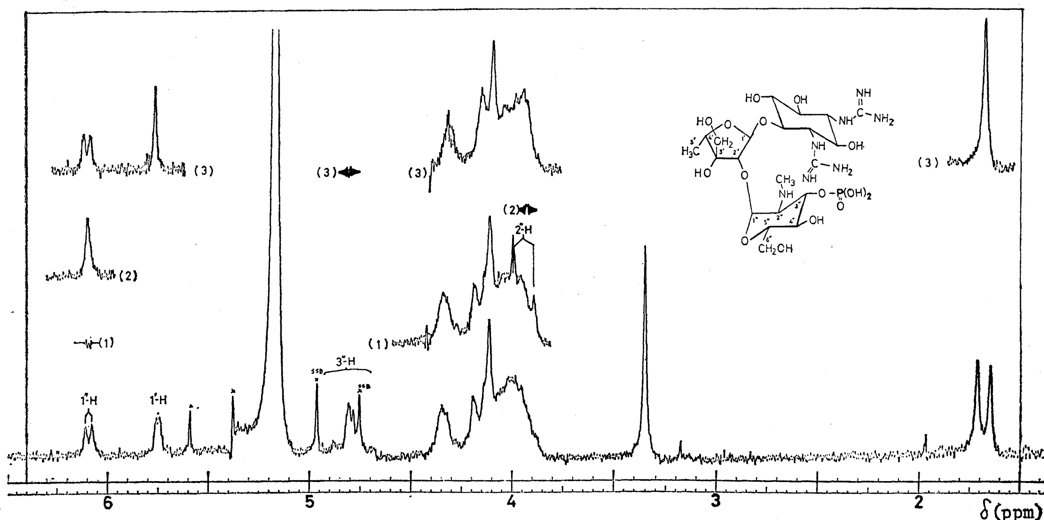


Fig. 6. PMR spectrum of dihydrostreptomycin-3″-phosphate (sulfate).



streptomycin-3″-phosphate (Fig. 6), 3″-H signal was observed at approximately δ 4.8 overlapping with the 2′-H and 4′-H in streptose. Irradiation at δ 4.8 caused the doublet of doublets signal at δ 3.94 (J=10.3, 3.0 Hz, 2′-H), the doublet signal at δ 5.74 (J=1.6 Hz, 1′-H) and the doublet signal at δ 1.68 (J=6.5 Hz, CH_3-C) to reduce to a doublet of J=10.3 Hz, a singlet and a singlet of methyl group, respectively. Although the splitting pattern of the 3″-H in dihydrostreptomycin-3″-phosphate was not observed, the 3″-H signal is shifted to a lower field by approximately 0.4 ppm when compared with dihydrostreptomycin. These results indicate that the phosphoryl group is attached at C-3 position in the N-methylglucosamine moiety.

Thus, the phosphorylated positions of three enzymatic inactivated antibiotics were confirmed by the application of double resonance techniques.

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