[Chem. Pharm. Bull. 26(4)1075—1082(1978)] UDC 547.918.02 : 576.851.098

# The Chemistry of Aminoglycoside Antibiotics from *Pseudomonas fluorescence*. I.[1,2] Isolation, Characterization and Partial Structure of P-2563(P) (Sorbistin $A_1$) and P-2563(A) (Sorbistin B)

KIYOSHI NARA, YASUHIRO SUMINO, KAZUYOSHI KATAMOTO, SHUN-ICHI AKIYAMA, and MITSUKO ASAI

*Central Research Division, Takeda Chemical Industries, Ltd.*[3]

(Received August 29, 1977)

Two aminoglycoside antibiotics active against Gram-positive and -negative bacteria, P-2563(P) (**1**) and P-2563(A) (**2**) were isolated from a culture broth of *Pseudomonas fluorescence* P-2563. It was found that **1** ($C_{15}H_{31}N_3O_9$) and **2** ($C_{14}H_{29}N_3O_9$) are acylaminoglycoside having the same new aminopolyol structure in their molecules. **1** and **2** showed low order of acute toxicity in mice.

**Keywords**——aminoglycoside antibiotics; *Pseudomonas fluorescence*; 4-amino-4-deoxy-D-glucose; new diamino-dideoxyhexitol; isolation

Recently the authors briefly reported the isolation and structure elucidation of two antibiotics P-2563(P) (**1**) and P-2563(A) (**2**) active against Gram-positive and -negative bacteria obtained from the culture broth of *Pseudomonas fluorescence* P-2563.[1,2] Tsukiura, *et al.*[4] independently reported the isolation and the structures of sorbistin $A_1$, $A_2$, B, C, and D, aminoglycoside antibiotics isolated from the culture broth of *P. sorbistii* nov. sp. The structures of **1** and **2** are identical with those of sorbistin $A_1$ and sorbistin B, respectively.

The authors' technique of structure elucidation, depending heavily upon proton magnetic resonance (PMR) decoupling experiments and $^{13}C$-nuclear magnetic resonance (NMR) spectra, is quite different from that of Tsukiura, *et al.* The authors now wish to deal with the details of the authors' studies on **1** and **2** in this series of papers. The purpose of the present note is to describe the isolation, characterization and partial structure of the two antibiotics.

The fermentation broth[5] was filtered with filter aid, and the filtrate was applied on a column of Amberlite IRC-50 ($NH_4^+$). The column was washed with water and then eluted with 1 N $NH_4OH$. The active eluates were combined, concentrated *in vacuo* and lyophilized.

The solid thus obtained was shown by thin-layer chromatography (TLC) to be a mixture of two bioactive components (designated as **1** and **2**).

The complex solid was separated into its components by ion-exchange column chromatography using Amberlite CG-50 ($NH_4^+$) developed with 0.08 N $NH_4OH$. Component **1** was eluted first, and component 2 was eluted successively. Each fraction was concentrated *in vacuo*, and applied on a column of Dowex 1×2 (OH), and developed with water. Eluates of each component were concentrated *in vacuo*, and the desired compounds were precipitated with acetone. **1** thus obtained was dissolved in water and the solution was adjusted to pH 6.5 with HCl. Dilution of the solution with propanol yielded monohydrochloride dihydrate

1) K. Nara, Y. Sumino, K. Katamoto, S. Akiyama, and M. Asai, *Chem. Lett.*, **1977**, 33.
2) K. Nara, K. Katamoto, S. Suzuki, S. Akiyama, and E. Mizuta, *Chem. Lett.*, **1977**, 229.
3) Location: *Juso-honmachi, Yodogawa-ku, Osaka 532, Japan.*
4) *a*) H. Tsukiura, M. Hanada, K. Saito, K. Fujisawa, T. Miyata, H. Koshiyama, and H. Kawaguchi, *J. Antibiot.*, **29**, 1137 (1976); *b*) M. Konishi, S. Kawata, T. Tsuno, K. Numata, H. Tsukiura, T. Naito, and H. Kawaguchi, *ibid.*, **29**, 1152 (1976).
5) Y. Sumino, K. Nara, and S. Akiyama, *J. Ferment. Technol.*, in press.

of **1**, as colorless prisms, $C_{15}H_{31}N_2O_9 \cdot HCl \cdot 2H_2O$. **2** thus obtained was dissolved in water and re-precipitated with acetone to a white powder of **2**, $C_{14}H_{29}N_3O_9$. The ratio of **1** to **2** was approximately 40: 60 in weight.

Three TLC systems, C-1, C-2 and C-3 were found suitable for differentiating the components (Table I).

Table I. TLC of P-2563(P)(**1**) and (A)(**2**)

| Solvent system[a] | *Rf*[b] | |
|---|---|---|
| | **1** | **2** |
| C-1 | 0.51 | 0.41 |
| C-2 | 0.22 | 0.10 |
| C-3 | 0.55 | 0.46 |

*a*) C-1: propanol–pyridine–AcOH–water (15: 10: 3: 12).
C-2: butanol–AcOH–water (3: 1: 1).
C-3: EtOAc–propanol–25%$NH_3$–water (1: 5: 1: 3).
*b*) Pre-Coated TLC Plates (cellulose, Merck).
detected by ninhydrin reagent.

The minimum inhibitory concentration (MIC) of **1** and **2** was determined by the serial two-fold agar dilution method against gram-positive and -negative bacteria as well as fungi. As shown in Table II, the intrinsic activity of **1** and **2** was moderate in terms of MIC values but their antibacterial spectra were relatively broad, inhibiting most of the bacterial strains tested. The activity of **1** is stronger than that of **2**.

Table II. Antimicrobial Spectrum of P-2563(P)(**1**) and (A)(**2**)

| Assay organism | Assay procedure[a] | Minimal inhibitory concentration (mcg/ml) | |
|---|---|---|---|
| | | P-2563(P) (**1**) | P-2563(A) (**2**) |
| *Bacillus subtilis* | I | 30 | 70 |
| *Staphylococcus aureus* | I | 50 | 200 |
| *Escherichia coli* | I | 30 | 50 |
| *Klebsiella pneumoniae* | I | 12.5 | 50 |
| *Pseudomonas aeruginosa* | I | 50 | 300 |
| *Proteus vulgaris* | I | 30 | 100 |
| *Proteus mirabilis* | I | 200 | 200 |
| *Mycobacterium* species | II | 200 | 200 |
| *Cryptococcus neoformans* | III | >500 | >500 |
| *Candida albicans* | III | >500 | >500 |
| *Asperigillus fumigatus* | III | >500 | >500 |
| *Microporum gypseum* | III | >500 | >500 |
| *Trichophton mentagrophytes* | III | >500 | >500 |

*a*) Procedures I to III mean the following:
Procedure I: Cultivated on heart infusion agar medium (Difco) at 37° for 24 hours.
Procedure II: Cultivated on heart infusion agar medium with 1% added glycerin at 37° for 48 hours.
Procedure III: Cultivated on glucose bouillon agar medium at 28° for 96 hours.

The acute toxicity ($LD_{50}$) of **1** and **2** in mice were found to be >400 mg/kg, respectively, by subcutaneous route.

Both **1** and **2** are basic. They were freely soluble in water, methanol and ethanol, but practically insoluble in butanol, acetone and other organic solvents. Both of them gave

positive reactions with ninhydrin, Molish-Udransky and Ehrlich reagents but were negative to Fehling and Sakaguchi reactions.

**1** had $[\alpha]_D^{20}$ +73.0° ($c$=1.0, $H_2O$), p$K_a'$ 7.2 and 9.6, and exhibited only end absorption in the ultraviolet (UV) spectrum. The infrared (IR) spectrum of **1** showed bands at 1640 and 1550 $cm^{-1}$ ascribable to an amide carbonyl group.

The PMR spectrum of **1** given in Fig. 1 exhibited the presence of an anomeric proton ($\delta$ 5.36 ppm) and a propionyl group.

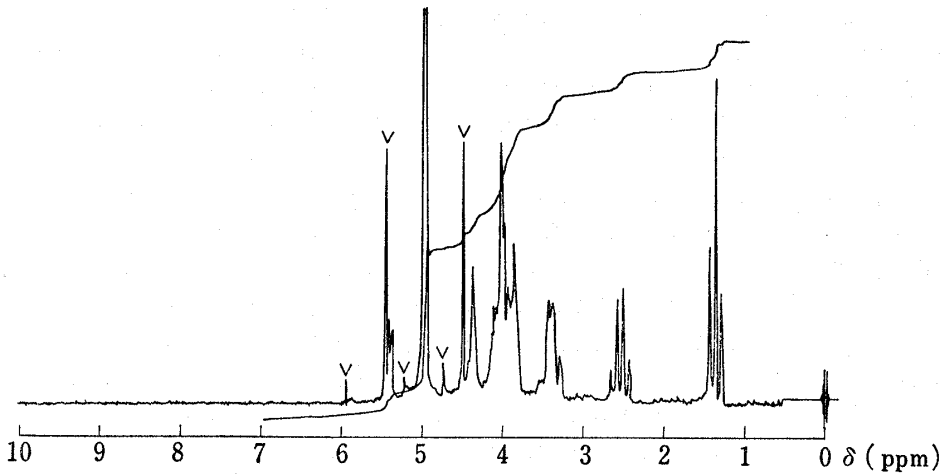



Fig. 1. PMR Spectrum of P-2563(P)(1)(100 MHz in $D_2O$)

**2** had $[\alpha]_D^{23}$ +76.1° ($c$=1.0, $H_2O$), p$K_a'$ 7.2 and 9.5, and exhibited only end absorption in the UV spectrum. The IR spectrum of **2** was nearly identical with that of **1**. The PMR spectrum of **2** (Fig. 2) differed from that of **1** and, instead of those of the propionyl group in **1**, proton signals assignable to an acetyl group was observed in **2**.

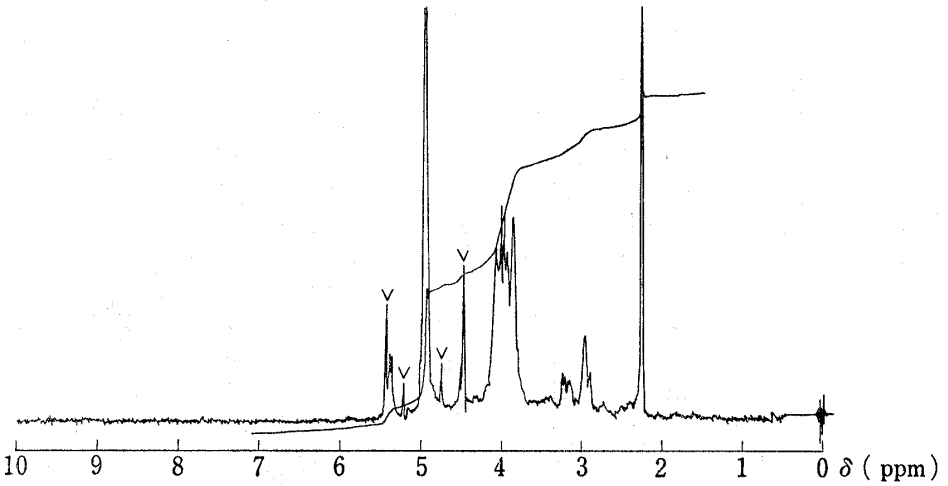



Fig. 2. PMR Spectrum of P-2563(A)(2)(100 MHz in $D_2O$)

Stepwise acetylation of **1** was first attempted to clarify the functional group of the compound. N-Acetylation of **1** with $Ac_2O$ in $H_2O$ gave the diacetate (**3**), $C_{19}H_{35}N_3O_{11}$ and acetylation of **3** with $Ac_2O$ in pyridine gave the octaacetate (**4**), $C_{31}H_{47}N_3O_{17}$. From these facts, it was assumed that two amino groups and six hydroxyl groups were present in **1**. The presence of two amino groups in **1** was also supported by next results. First, when **1** was treated with $p$-methoxybenzylaldehyde and the resultant Shiff's base was reduced with $NaBH_4$ and the reduced product was acetylated with $Ac_2O$ in pyridine, the di-N-($p$-methoxy-

benzyl) octaacetate (**5**), $C_{47}H_{63}N_3O_{19}$, was obtained. Second, when aqueous solution of **1** was treated with benzyl chloride, and the resultant product was treated with $Ac_2O$ in pyridine, the tetra-N-benzyl-hexaacetate (**6**), $C_{55}H_{67}N_3O_{15}$, was obtained.

An alkaline hydrolysis of **1** yielded sodium propionate (**7**) and the depropionyl derivative (**8**), $C_{12}H_{27}N_3O_8$, $pK_a'$ 8.7 (2 mol) and 6.5 (1 mol). N-Acetylation of **8** with $Ac_2O$ in $H_2O$ afforded the tri-N-acetate (**9**). Acetylation of **9** with $Ac_2O$ in pyridine afforded the nonaacetate (**10**). Comparison of the PMR spectrum of **10** with that of **4** suggested that the propionyl group in **4** had been replaced by an acetyl group.

From the above observations, the three nitrogen atoms in **1** are characterized as one amido carbonyl and two amino groups and the nine oxygen atoms in **1** are also characterized as six alcoholic hydroxyls, one amido carbonyl and two ether oxygens. Above assignment is consistent with the fact that the unsaturation number of **1** is two (amido carbonyl and ring ether).

N-Acetylation of **2** with $Ac_2O$ in $H_2O$ gave the diacetate (**11**), $C_{16}H_{31}N_3O_{10}$ and acetylation of **11** with $Ac_2O$ in pyridine gave the octaacetate (**12**), $C_{30}H_{45}N_3O_{17}$. **12** thus obtained was found to be identical with the nonaacetate of **8** in all respects. Thus, it was concluded that **2** is a compound having an acetyl group instead of a propionyl group in **1**.

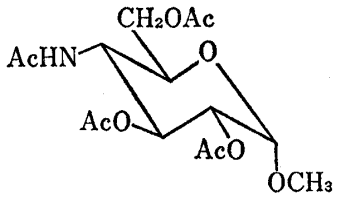
Clear information came from methanolysis of **1** and **8** with MeOH–HCl, which afforded the same methylglycosides of aminosugar anomers (**13a,b**), $C_7H_{15}NO_5$, and ninhydrin-positive substance (**14**). **13** was acetylated with $Ac_2O$ in pyridine and the acetates were chromatographed on silica gel to give $\alpha$(or $\beta$)-methylglycoside tetraacetate (**15a**), $C_{15}H_{23}NO_9$, $[\alpha]_D^{23}$ +156° ($c$=1.0, $CHCl_3$) and $\beta$(or $\alpha$)-methylglycoside tetraacetate (**15b**), $C_{15}H_{23}NO_9$, $[\alpha]_D^{23}$ +0.5° ($c$=1.0, $CHCl_3$).

The PMR spectrum of **15a** (Table III) showed an anomeric proton ($\delta$ 4.94, d, $J$=4 Hz, H-1) and two sidechain methylene protons ($\delta$ 4.18, d, $J$=4.0 Hz, H-6a, 6b). The doublet

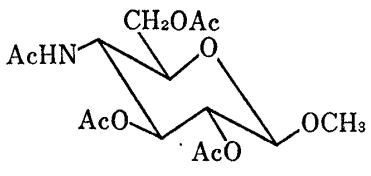
Table III. PMR Spectra of Methylglycoside Tetraacetate (**15a, 15b**) (100 MHz in $CDCl_3$)

| | | H 3 | H 1 | H 2 | H 6a,b | H 4 | H 5 | Ac | $OCH_3$ | NH on C-4 |
|---|---|---|---|---|---|---|---|---|---|---|
| **15a** | $\delta$ (ppm) | 5.28 | 4.94 | 4.86 | 4.18 | 4.15 | 3.80 | 1.95<br>2.05 | 3.36 | 5.92 |
| | | t | d | d.d | d | q | d.t | s | s | d |
| | | 1H | 1H | 1H | 2H | 1H | 1H | 3H×4 | 3H | 1H |
| | $J$ (Hz) | $J_{2-3}$ 10<br>$J_{3-4}$ 10 | $J_{1-2}$ 4 | $J_{1-2}$ 4<br>$J_{2-3}$ 10 | $J_{5-6}$ 4 | $J_{3-4}$ 10<br>$J_{4-5}$ 10<br>$J_{4-NH}$ 10 | $J_{4-5}$ 10<br>$J_{5-6}$ 4 | | | $J_{4-NH}$ 10 |
| **15b** | $\delta$ (ppm) | 5.11 | 4.38 | 4.93 | 4.20 | 4.08 | 3.65 | 1.90<br>2.10 | 3.46 | 5.82 |
| | | t | d | d.d | d | q | d.t | s | s | d |
| | | 1H | 1H | 1H | 2H | 1H | 1H | 3H×4 | 3H | 1H |
| | $J$ (Hz) | $J_{2-3}$ 10<br>$J_{3-4}$ 10 | $J_{1-2}$ 7.4 | $J_{1-2}$ 7.4<br>$J_{2-3}$ 10 | $J_{5-6}$ 4 | $J_{3-4}$ 10<br>$J_{4-5}$ 10<br>$J_{4-NH}$ 10 | $J_{4-5}$ 10<br>$J_{5-6}$ 4 | | | $J_{4-NH}$ 10 |

Abbreviation: s=singlet, d=doublet, t=triplet, q=quartet, d.d=double doublet, d.t=double triplet.




**15a**




**15b**

at $\delta$ 5.92 ($J$=10 Hz, AcNH–) diminished on exchange with $D_2O$. The fact also revealed the expected simplification in the quartet pattern at $\delta$ 4.15 assignable to the AcNH–CH– proton. When the double triplet centered at $\delta$ 3.80 was irradiated, the H-6a, 6b doublet was collapsed into singlet, and, at the same time, the triplet at $\delta$ 4.15 (H-4) was collapsed into a doublet ($J$=10 Hz), then, when this triplet at $\delta$ 4.15 (H-4) was irradiated, the double triplet at $\delta$ 3.80 (H-5) became a triplet ($J$=4 Hz) and, at the same time, the triplet ($J$=10 Hz) at $\delta$ 5.28 (H-3) formed a doublet ($J$=10 Hz). Irradiation of this triplet at $\delta$ 5.28 (H-3) caused collapse of both the triplet at $\delta$ 4.15 (H-4) into a doublet ($J$=10 Hz) and the double doublet at $\delta$ 4.86 ($J$=4 Hz and 10 Hz, H-2) into a doublet ($J$=4 Hz). Thus, the chemical shifts of each proton and the coupling constants obtained are shown in Table III.

On the basis of these data, the authors assigned methyl 4-amino-4-deoxy-glucopyranosides to **15a** and **15b**. Relationship between **15a** and **15b** was also confirmed by scrutinizing the PMR spectra.

The coupling constants of the methine protons H-2 through H-5 ($J$=10 Hz) in **15a** indicated that these methine protons are all axial and the coupling constant ($J$=4 Hz) between H-1 and H-2 indicated that these protons are equatorial. The magnitude of the coupling constants in **15b** indicated that the methine protons H-1 through H-5 are all axial, indicating that **15b** is the anomer of **15a**.

The absolute configuration of **15a** and **15b** was assumed to be methyl 4-amino-4-deoxy-$\alpha$-D-glucopyranoside tetraacetate (C1), methyl 4-amino-4-deoxy-$\beta$-D-glucopyranoside tetraacetate (C1), respectively, from the above evidence, specific rotations and Hudson's rule.[6] The IR spectrum, PMR spectrum and specific rotation of **15a** were identical with those of methyl 4-amino-4-deoxy-$\alpha$-D-glucopyranoside tetraacetate reported by Reist[7] and Agahigian.[8]

**14** was shown to be an aminohexitol. **14** was purified by column chromatography on Amberlite CG-50, and crystallized from aqueous acetone, $C_6H_{16}N_2O_4$, $[\alpha]_D^{20}$ −1.85° ($c$=1.0, $H_2O$), p$K_a'$ 8.6 (2 mol). It was soluble in water, methanol, and ethanol, and sparingly soluble in acetone, EtOAc and organic solvents. It gave positive reactions with $KMnO_4$, but was negative with Fehling and Molish-Udransky reagents. The UV spectrum of **14** in aqueous solution showed only end absorption, and the IR spectrum indicated the presence of hydroxyl or ether function (1000—1100 $cm^{-1}$). The PMR spectrum (in $D_2O$) of **14** indicated eight proton signals assignable to N,O-methine and N,O-methylene (2.8—4.1 ppm). N-Acetylation of **14** with $Ac_2O$ in $H_2O$ gave the di-N-acetate (**16**), $C_{10}H_{20}N_2O_6 \cdot H_2O$ and acetylation of **16** with $Ac_2O$ in pyridine gave the hexaacetate (**17**), $C_{18}H_{28}N_2O_{10}$, MS $m/e$: 432 ($M^+$), 433 ($M^+$+1). From above results, it is suggested that **14** has a diaminodideoxyhexitol structure.

The above studies have outlined the antimicrobial properties and chemical features of **1** and **2** which have been shown to be aminohexitol glycosides carrying a propionyl and an acetyl group, respectively. Further structure elucidation of **1** and **2** is dealt with in the subsequent papers.

## Experimental

The following instruments were used for obtaining physical data. Melting point, Yanagimoto's microscope hot stage (uncorrected); UV spectra, Hitachi spectrophotometer Model 124 in water; IR spectra, Hitachi grating infrared spectrophotometer in KBr; PMR spectra (tetramethylsilane as internal standard, $\delta$ value), Varian HA-100 spectrometer in $CDCl_3$. TLC was performed on pre-coated TLC plates (silica gel, Merck) and following solvent systems were used; solvent S-1 (propanol–pyridine–AcOH–$H_2O$=15: 10: 3: 12), solvent S-2 (butanol–AcOH–$H_2O$=3: 1: 1), solvent S-3 (EtOAc).

**Isolation of P-2563(P) (1) and P-2563(A) (2)**——Fermentation broth (32 l) was filtered with filter aid, and the filtrate (30 l) was applied to a column of Amberlite IRC-50 ($NH_4^+$) (3 l). The column was washed

6) G.S. Hudson, *J. Am. Chem. Soc.*, **31**, 66 (1909).
7) E.J. Reist, R.R. Spencer, D.F. Calkins, B.R. Baker, and L. Goodman, *J. Org. Chem.*, **30**, 2312 (1965).
8) H. Agahigian, G.D. Vickers, M.H. von Saltza, J. Reid, A.I. Cohen, and H. Gassthier, *J. Org. Chem.*, **30**, 1085 (1965).

with water (9 l) and eluted with 1 N $NH_4OH$. The eluates were monitored by TLC (S-1, detected by ninhydrin reagent and bioautography against *Escherichia coli* IFO 3044). The active eluates (6 l) were combined, concentrated *in vacuo* and lyophilized. The crude solid (30 g) thus obtained was dissolved in water (300 ml) and applied to a column of Amberlite CG-50 ($NH_4^+$, I type) (600 ml). The column was washed with water and then eluted with 0.08 N $NH_4OH$, the eluate being collected in 200 ml fractions. The fractions No. 10—21 containing **1** and the fractions No. 22—30 containing **2** were pooled. The combined fractions containing **1** were concentrated *in vacuo*; the ammonia was substantially removed; and the residue was dissolved in water (50 ml). The solution was run onto a column of Dowex 1×2 (OH) (100 ml), and it was eluted with water successively. The effluent and the eluate were combined, and concentrated *in vacuo*. The concentrate was diluted with acetone; precipitates whereby obtained were dried to give **1** (4.0 g) as a white powder, mp 105—115° (dec.), molecular weight (vapor pressure osmometry): 420. The PMR spectrum is shown in Fig. 1. *Anal.* Calcd. for $C_{15}H_{31}N_3O_9$: C, 45.34; H, 7.81; N, 10.58. Found: C, 45.65; H, 7.89; N, 10.79.

The combined fractions containing **2** were concentrated *in vacuo* and to the concentrate was added acetone to give a white powder of **2** (2.5 g), mp 148—150° (dec.). The PMR spectrum is shown in Fig. 2. *Anal.* Calcd. for $C_{14}H_{29}N_3O_9$: C, 43.68; H, 7.57; N, 10.97. Found: C, 43.68; H, 7.59; N, 10.43.

**P-2563(P) Monohydrochloride**——**1** (5 g) was dissolved with water (10 ml) and the solution was neutralized to pH 6.5 with HCl and to the solution was added propanol (30 ml). The mixture was kept standing in a refrigerator for 24 hr to give colorless prisms of monohydrochloride of **1** (2.5 g), mp 98—108° (dec.). $[\alpha]_D^{23}$ +60.3° ($c$=1.0, $H_2O$). *Anal.* Calcd. for $C_{15}H_{31}N_3O_9 \cdot HCl \cdot 2H_2O$: C, 38.34; H, 7.67; N, 8.95; Cl, 7.56; O, 37.49. Found: C, 38.49; H, 7.66; N, 8.98; Cl, 7.28; O, 37.59.

**P-2563(P) Di-N-acetate (3)**——**1** (1 g) in water (7 ml) was acetylated with $Ac_2O$ (3.5 ml) for 20 hr. The reaction mixture was evaporated *in vacuo* to dryness. Crystallization of the residue with MeOH–EtOAc gave white prisms, mp 156—158° (dec.), $[\alpha]_D^{23}$ +108° ($c$=1.0, $H_2O$). IR $\nu_{max}^{KBr}$: 3400, 1650, 1545, 1065, 1020. PMR ($D_2O$) $\delta$: 1.16 (3H, t, $J$=7 Hz, $-CH_2-C\underline{H}_3$), 2.03 (3H, s, $-CO-C\underline{H}_3$), 2.08 (3H, s, $-CO-C\underline{H}_3$), 2.34 (2H, q, $J$=7 Hz, $-CO-C\underline{H}_2-CH_3$), 2.9—4.3 (14H, N,O-methine, N,O-methylene), 5.23 (1H, d, $J$=3.5 Hz, anomeric proton). *Anal.* Calcd. for $C_{19}H_{35}N_3O_{11} \cdot 1/2H_2O$: C, 46.53; H, 7.35; N, 8.57. Found: C, 46.26; H, 7.39; N, 8.49.

**P-2563(P) N,O-Octaacetate (4)**——**3** (1 g) in pyridine (7 ml) was acetylated with $Ac_2O$ (3.5 ml) for 20 hr. The resulting product (1.3 g) was crystallized from EtOAc–hexane to yield white needles, mp 199—201° (dec.). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3350, 2950, 1745, 1630, 1510, 1370, 1240, 1060, 1030. MS $m/e$: 733 ($M^+$). *Anal.* Calcd. for $C_{31}H_{47}N_3O_{17}$: C, 50.75; H, 6.41; N, 5.73. Found: C, 50.63; H, 6.41; N, 5.71.

**P-2563(P) Di-N-(*p*-methoxybenzyl)-N,O-octaacetate (5)**——To **1** (4 g, 40 mmol) in water (50 ml), NaOH (0.6 g) in water (2 ml) was added dropwise under stirring and, then *p*-anisaldehyde (6.5 g, 50 mmol) was added and stirred for further 30 min at room temperature. The reaction mixture was evaporated *in vacuo* almost to dryness. The residue was dissolved in EtOH (100 ml) and the solution was diluted with ethyl ether (500 ml) to give a white precipitate. The precipitate was dried *in vacuo* over $P_2O_5$. The powder was dissolved in a mixture of tetrahydrofuran–MeOH (3: 2) (200 ml) and to the solution sodium borohydride ($NaBH_4$) (1 g) was added and the mixture was stirred for 2 hr at room temperature. The excess $NaBH_4$ was decomposed by acetone (50 ml) and the mixture was evaporated *in vacuo* to dryness to leave a crude powder (2.5 g). The crude powder was dissolved in water (50 ml), passed through an Amberlite CG-50 ($NH_4^+$) column (500 ml) and eluted with a linear gradient (water to 1.5 N $NH_4OH$, each 2 l). The eluates were monitored by TLC (S-2, detected by Molish-Udransky reagent) and the desired fractions were combined and concentrated *in vacuo* to afford a white solid of di-N-(*p*-methoxybenzyl) derivative (2.1 g).

**Di-N-(*p*-methoxybenzyl) Derivative of 1**——Calcd. for $C_{31}H_{47}N_3O_{11} \cdot 3H_2O$: C, 53.83; H, 7.67; N, 6.08. Found: C, 53.86; H, 7.45; N, 5.96.

The product (2 g) in pyridine (14 ml) was acetylated with $Ac_2O$ (7 ml) for 20 hr at room temperature. The resulting crude powder (3 g) was dissolved in $CHCl_3$ (30 ml), passed through a silica gel column (300 ml); the column was eluted with $CHCl_3$–acetone (5: 1) to give a white powder of **5** (2.5 g), $[\alpha]_D^{24}$ +46.5° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3350, 2950, 1745, 1630, 1510, 1370, 1240, 1030. PMR ($CDCl_3$) $\delta$: 1.20 (3H, t, $J$= 7 Hz, $-CH_2-C\underline{H}_3$), 1.75—2.12 (3H×8, s, $CO-C\underline{H}_3$), 2.18 (2H, q, $J$=7 Hz, $-CO-C\underline{H}_2-CH_3$), 2.8—5.72 (19H, N,O-methine, N,O-methylene), 3.76, 3.82 (3H×2, s, $O-CH_3$), 6.66—7.20 (8H, m, benzene ring proton). *Anal.* Calcd. for $C_{47}H_{63}N_3O_{19}$: C, 57.96; H, 6.47; N, 4.32. Found: C, 57.31; H, 6.48; N, 4.37.

**P-2563(P) Tetra-N-benzyl-hexa-O-acetate (6)**——A mixture of **1** (2 g, 5 mmol), 50% aqueous EtOH (100 ml) saturated with $NaHCO_3$ and benzyl chloride (9.2 ml, 80 mmol) was refluxed for 5 hr. The insoluble material was filtered off and the filtrate was evaporated *in vacuo* to dryness. The crude product dissolved in EtOAc (120 ml) was charged on a silica gel column (300 ml). The column was eluted with EtOAc–acetone (5: 1). Appropriate fractions were combined and evaporated *in vacuo* to give a white powder of tetra-N-benzyl derivative of **1** (2.5 g).

**Tetra-N-benzyl Derivative of 1**——*Anal.* Calcd. for $C_{43}H_{55}N_3O_9 \cdot H_2O$: C, 66.58; H, 7.35; N, 5.42. Found: C, 66.33; H, 7.24; N, 5.12.

The product (2 g) in pyridine (14 ml) was acetylated with $Ac_2O$ (7 ml) for 20 hr at room temperature. From a resulting mixture a crude powder (2.5 g) was obtained. The product was dissolved in $CHCl_3$ (25 ml)

and applied to a silica gel column (300 ml); the column was eluted with $CHCl_3$–acetone (5: 1). The desired fractions were combined and evaporated to dryness. The crude product was crystallized with EtOAc–hexane to white needles of **6** (2 g), mp 80° (dec.), $[\alpha]_D^{28}$ +64.0° ($c$=1.0, $CHCl_3$). PMR ($CDCl_3$) $\delta$: 1.04 (3H, t, $J$=7 Hz, –CO–$CH_2$–$C\underline{H}_3$), 2.03 (2H, q, $J$=7 Hz, –CO–$C\underline{H}_2CH_3$), 1.90—2.10 (3H×6, s, –CO–$C\underline{H}_3$), 2.33, 2.67 (1H×2, m, N-methine), 3.00—5.26 (20H, N,O-methine, N,O-methylene), 5.60 (1H, m, O-methine), 7.10—7.50 (20H, benzene ring proton). *Anal.* Calcd. for $C_{55}H_{67}N_3O_5$: C, 65.41; H, 6.64; N, 4.16. Found: C, 65.23; H, 6.81; N, 4.22.

**Sodium Propionate (7) (Alkaline Hydrolysis of 1)**——**1** (2 g, 5 mmol) in 0.5 N NaOH (40 ml) was refluxed for 2 hr. The reaction mixture was passed through a Dowex 50 ($H^+$) column (70 ml). The column was washed with water. The effluent and the washing were combined, neutralized to pH 7.0 with 1 N NaOH and evaporated to dryness to leave a white powder of **7** (425 mg). The IR spectrum of **7** was identical with that of authentic sodium propionate. The retention time (4.5 min) of the acid obtained from **7** was also identical with that of authentic propionic acid in gas chromatography (Chromosorb W-AW/SE-30; column temp., programmed at a rate of 10°/min from 60°; injection temp., 280°). *Anal.* Calcd. for $C_3H_5O_2Na$: C, 37.5; H, 5.20; Na, 24.0. Found: C, 37.3; H, 5.32; Na, 24.0.

**Depropionyl Derivative of 1 (8) (Alkaline Hydrolysis of 1)**——The above Dowex 50 ($H^+$) column was successively eluted with 1 N $NH_4OH$. Evaporation of the eluate left a crude powder (1.55 g). The crude powder was dissolved in water (30 ml) and applied to an Amberlite CG-50 ($NH_4^+$) column (300 ml); the column was eluted with a linear gradient (water to 2 N $NH_4OH$, each 1 l). The eluates were monitored by TLC (S-1, detected by ninhydrin reagent). Fractions having an $Rf$ 0.10 were combined, concentrated and charged on a Dowex 1×2 ($OH^-$) column (100 ml) and eluted with water. The eluates were combined, evaporated to give a white powder of **8** (1.40 g), mp 130—140° (dec.), $[\alpha]_D^{28}$ +82.4° ($c$=1.0, $H_2O$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400, 1600, 1160—1020. PMR ($D_2O$) $\delta$: 2.8—4.6 (14H, N,O-methine, N,O-methylene), 5.21 (1H, d, $J$=3.0 Hz, anomeric proton). *Anal.* Calcd. for $C_{12}H_{27}N_3O_8$: C, 42.23; H, 7.92; N, 12.32. Found: C, 42.08; H, 7.90; N, 12.22.

**Tri-N-acetate of 8 (9)**——**8** (1 g) in water (10 ml) was acetylated with $Ac_2O$ (3 ml) for 16 hr at room temperature. The reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved with water (10 ml) and charged on a Dowex 50 (H) column (20 ml) and eluted with water. The eluates were combined and evaporated to dryness *in vacuo*. The product was crystallized from MeOH–EtOAc to white prisms (**9**), mp 235—238° (dec.), $[\alpha]_D^{28}$ +115° ($c$=1.0, $H_2O$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400, 1650, 1545, 1375, 1065, 1020. PMR ($D_2O$) $\delta$: 1.93—2.14 (27H, s, CO–$C\underline{H}_3$), 2.74 (1H, m, N-methine), 3.94—5.14 (13H, N,O-methine, N,O-methylene), 5.54 (1H, m, O-methine), 6.26—6.48 (2H, –N$\underline{H}$), 6.98 (1H, –N$\underline{H}$). *Anal.* Calcd. for $C_{18}H_{33}N_3O_{11}$: C, 46.25; H, 7.07; N, 8.99. Found: C, 46.05; H, 7.17; N, 8.78.

**N,O-Nonaacetate of 9 (10)**——**9** (1 g) in pyridine (7 ml) was acetylated with $Ac_2O$ (3.5 ml) for 20 hr at room temperature. The crude product (1.55 g) was crystallized from EtOAc–hexane to white prisms (**10**), mp 189—191° (dec.), $[\alpha]_D^{27}$ +69.0° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3430, 3300, 1750, 1690, 1660, 1540, 1375, 1230, 1065, 1040, 600. PMR ($CDCl_3$) $\delta$: 1.94—2.16 (3H×9, s, CO–$C\underline{H}_3$), 2.77 (1H, m, N-methine), 3.8—5.15 (14H, N,O-methine, N,O-methylene), 5.48 (1H, m, O-methine), 6.30—7.10 (3H, –N$\underline{H}$–). MS $m/e$: 719 ($M^+$). *Anal.* Calcd. for $C_{30}H_{45}N_3O_{17}$: C, 50.01; H, 6.26; N, 5.84. Found: C, 49.87; H, 6.26; N, 5.74.

**P-2563(A) Di-N-acetate (11)**——**2** (1 g) in water (10 ml) was acetylated with $Ac_2O$ (3 ml) for 16 hr at room temperature. The reaction mixture was evaporated to dryness. The residual product dissolved in water (5 ml) was charged on a Dowex 50 (H) column (20 ml) and eluted with water. The eluate was evaporated to dryness. The product was crystallized from MeOH–EtOAc to white prisms of **11** (0.7 g), mp 235—238° (dec.), $[\alpha]_D^{28}$ +114.5° ($c$=1.0, $H_2O$). *Anal.* Calcd. for $C_{18}H_{33}N_3O_{11}$: C, 46.25; H, 7.07; N, 8.99. Found: C, 46.20; H, 7.09; N, 8.80.

**P-2563(A) N,O-Octaacetate (12)**——**11** (1 g) in pyridine (7 ml) was acetylated with $Ac_2O$ (3.5 ml) for 20 hr at room temperature. The resulting crude product (1.53 g) was crystallized from EtOAc–hexane to white needles of **12**, mp 189—191° (dec.), $[\alpha]_D^{27}$ +69.1° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3440, 3300, 1750, 1690, 1660, 1540, 1375, 1230, 1065, 1040, 600. PMR ($CDCl_3$) $\delta$: 1.94—2.16 (3H×9, s, CO–$C\underline{H}_3$), 2.77 (1H, m, N-methine), 3.8—5.15 (14H, N,O-methine, N,O-methylene), 5.48 (1H, m, O-methine), 6.30—7.10 (3H, –N$\underline{H}$–). MS $m/e$: 719 ($M^+$). *Anal.* Calcd. for $C_{30}H_{45}N_3O_{17}$: C, 50.01; H, 6.26; N, 5.84. Found: C, 49.98; H, 6.25; N, 5.80.

**Methyl Aminoglycosides (13a, b) (Methanolysis of 1)**——**1** (20 g) in MeOH saturated with HCl (900 ml) was refluxed for 12 hr. The reaction mixture was concentrated to leave a crude syrup. The crude syrup dissolved in water (100 ml) was passed through an Amberlite IR 45 (OH) column and the eluates containing the glycoside mixture were successively passed through an Amberlite CG-50 ($NH_4^+$) column (600 ml) and the column was washed with water. The column was then eluted with a linear gradient (water to 1.5 N $NH_4OH$, each 1.3 l). The eluates were monitored by TLC (S-1, detected by ninhydrin reagent). The fractions showing an $Rf$ 0.7 (substance as a major component) were combined and evaporated *in vacuo* to a hygroscopic mixture (3.6 g). *Anal.* Calcd. for $C_7H_{15}NO_5$: C, 43.52; H, 7.77; N, 7.25. Found: C, 43.14; H, 7.81; N, 7.08.

**Diaminohexitol (14) (Methanolysis of 1)**——In the above experiment, the fractions showing an $Rf$ 0.25 were combined and evaporated *in vacuo* to a crude **14** (3.34 g). From the fractions showing an $Rf$ 0.1, crude **8** (6.45 g) was obtained. The crude **14** (3 g) dissolved in water (20 ml) was charged on a Dowex 1×2 (OH)

column (150 ml) and eluted with water. The eluates were concentrated *in vacuo* to a syrup. The syrup was kept standing in a refrigerator for 24 hr to afford white needles of **14** (2.02 g), mp 108—110°. PMR ($D_2O$) $\delta$: 2.8—4.1 (8H, N,O-methine, N,O-methylene). *Anal.* Calcd. for $C_6H_{16}N_2O_4$: C, 40.0; H, 8.90; N, 15.55. Found: C, 39.88; H, 9.02; N, 15.45.

**Methyl Aminoglycoside Tetraacetates (15a, 15b)**——The mixture of methyl aminoglycoside (**13a, b**) (3 g) in pyridine (30 ml) was acetylated with $Ac_2O$ (15 ml) for 24 hr at room temperature. The crude product was dissolved in EtOAc–benzene (1: 2) (60 ml) and the solution was applied to a silica gel column (1 l). The The column was eluted with EtOAc–benzene (3: 1), the eluate being collected in 50 ml fractions. The fractions were monitored by TLC (S-3, detected by ninhydrin reagent). The fractions showing an *Rf* 0.50 were collected. The crude product was crystallized from EtOAc–hexane to white prisms of **15a** (1.1 g), mp 138—140°, $[\alpha]_D^{23}$ +156° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3350, 1740, 1670, 1540, 1385, 1250, 1070—1040, 917, 844, 770, 620, 560, 500. The PMR spectrum is shown in Table III. MS *m/e*: 361 ($M^+$), 330 ($M^+-OCH_3$). *Anal.* Calcd. for $C_{15}H_{23}NO_9$: C, 49.86; H, 6.42; N, 3.88. Found: C, 49.88; H, 6.34; N, 3.85.

The fractions showing an *Rf* 0.45 were combined, evaporated *in vacuo* to obtain a crude product of **15b** (1.0 g). The crude **15b** was rechromatographed on a silica gel column, eluted with EtOAc–benzene (3: 1). The product thus obtained was crystallized from EtOAc–hexane to white prisms of **15b** (0.74 g), mp 202°, $[\alpha]_D^{23}$ +0.5° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3350, 1750, 1670, 1560, 1390, 1250, 1070—1050, 910, 882, 700, 610, 510. The PMR spectrum is shown in Table III. MS *m/e*: 361 ($M^+$), 330 ($M^+-OCH_3$). *Anal.* Calcd. for $C_{15}H_{23}NO_9$: C, 49.86; H, 6.42; N, 3.88. Found: C, 49.85; H, 6.40; N, 3.83.

**Di-N-acetate of 14 (16)**——**14** (1.2 g) in water (20 ml) was acetylated with $Ac_2O$ (7 ml) for 8 hr at room temperature. The product (1.4 g) was crystallized from water–acetone–hexane to white needles of **16** (1.0 g), mp 87—89°, $[\alpha]_D^{23}$ +14.5° ($c$=1.0, $H_2O$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3450—3300, 1645, 1620, 1560, 1470, 1080, 1040. PMR ($D_2O$) $\delta$: 2.20, 2.18 (3H×2, s, CO–$CH_3$), 3.20—4.24 (8H, N,O-methine, N,O-methylene). *Anal.* Calcd. for $C_{10}H_{22}N_2O_7 \cdot H_2O$: C, 42.55; H, 7.80; N, 9.93. Found: C, 42.80; H, 7.94; N, 9.95.

**N,O-Hexaacetate of 14 (17)**——**16** (200 mg) in pyridine (1.4 ml) was acetylated with $Ac_2O$ (0.7 ml) for 20 hr at room temperature. The crude product (300 mg) was crystallized from EtOAc–hexane to white prisms of **17** (210 mg), mp 115—116°, $[\alpha]_D^{23}$ +55° ($c$=1.0, $CHCl_3$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3360, 1750, 1660, 1545, 1380, 1240, 1065. MS *m/e*: 432 ($M^+$). *Anal.* Calcd. for $C_{18}H_{28}N_2O_{10}$: C, 50.0; H, 6.48; N, 6.48. Found: C, 49.64; H, 6.47; N, 6.49.

**Acknowledgement** The authors would like to thank Drs. E. Ohmura, M. Isono, H. Fukuda, K. Mizuno, Y. Nakao and T. Kishi of this division for their advice and encouragement. Thanks are also due to Messrs. M. Deguchi and K. Asai for technical assistance.