BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 39 1244—1248 (1966)

Studies of Aminosugars. XII. The Absolute Structure of Kanamycin as Determined by a Copper Complex Method*1

By Sumio UMEZAWA, Kuniaki TATSUTA and Tsutomu TSUCHIYA

Department of Applied Chemistry, Faculty of Engineering, Keio University, Koganei-shi, Tokyo

(Received October 23, 1965)

N, *N'*-Dimethyl-di-*O*-methyldeoxystreptamine has been prepared from kanamycin through the hydrolysis of the totally-methylated product of *O*-(3-acetamido-3-deoxy-α-D-glucopyranosyl)-*N*, *N'*-diacetyldeoxystreptamine. On the other hand, *N*, *N'*-dimethyl-4, 5, 6-tri-*O*-methyldeoxystreptamine has been prepared by hydrolysis of the totally-methylated product of *N*, *N'*-diacetyldeoxystreptamine. The shifts in molecular rotations of the both products have then been determined, with solutions of tetramminecopper(II) sulfate, ammoniacal cuprous chloride and Cupra B being substituted for water as the solvent. By the application of the generalizations described in the foregoing paper, it has been found that the former product includes a pair of adjacent methylamino and hydroxyl groups and that the projected angle between them is about +60°. These findings led to the conclusion that the free hydroxyl group in the former product is attached to the C-6 of deoxystreptamine, indicating that, in kanamycin, 6-amino-6-deoxy-α-D-glucosyl and 3-amino-3-deoxy-α-D-glucosyl moieties are attached to the C-4 and C-6 of deoxystreptamine respectively.

In earlier papers, we reported the results of the methylation[1] and partial hydrolysis studies[2] of kanamycin; thus the structure[1a] of kanamycin was deduced, with the reservation that the absolute sequence of the substitution of 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose at the C-4 and C-6 of deoxystreptamine remained undetermined.

Recently, Hichens and Rinehart[3] successfully applied the cuprammonium method of Reeves[4] to the investigation of the absolute structure of neomycins, paromomycins and kanamycins, by measuring the shift of the molecular rotation, $\Delta[M]_{\text{Cupra B}}$, of *N*, *N'*-diacetyl-6-*O*-methyldeoxystreptamine obtained from neomycin B and C and from paromomycin. Tatsuoka et al.[5] used the same method to elucidate the absolute configuration of kanamycin, measuring the $\Delta[M]_{\text{Cupra B}}$ of tri-*N*-acetyl-kanosaminyl-deoxystreptamine.

The work of Reeves, using "Cupra B," provides a tool for the determination of the stereochemical relationship between adjacent hydroxyl groups. In the preceding paper[6], the determination of $\Delta[M]_{\text{TACu}}$*2 has been introduced as a method for the revelation of the stereochemical relationship between adjacent amino and hydroxyl groups in a molecule of a six-membered chair form; this method involves measuring the shift in the opticalrotation when a tetramminecopper(II) sulfate solution (TACu) is substituted for water as the solvent.

The present report will describe the determination of the absolute structure of kanamycin by the use of TACu in addition to an ammoniacal cuprous chloride solution and "Cupra B." For this purpose, *N*, *N'*-dimethyl-di-*O*-methyldeoxystreptamine (IV) has been prepared, starting from kanamycin, by the route shown in Scheme I(1). Additionally, *N*, *N'*-dimethyl-tri-*O*-methyldeoxystreptamine (VII) has been prepared from deoxystreptamine as a reference substance (Scheme I (2)).

(1) Kanamycin → 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-deoxystreptamine (I) → 6-*O*-(3-acetamido-3-deoxy-α-D-glucopyranosyl)-*N*, *N'*-diacetyl-deoxystreptamine (II) → 6-*O*-[3-deoxy-2, 4, 6-tri-*O*-methyl-3-(*N*-methylacetamido)-α-D-glucopyranosyl]-*N*,*N'*-diacetyl-*N*, *N'*-dimethyl-4, 5-di-*O*-methyl-deoxystreptamine (III) → *N*, *N'*-dimethyl-4, 5-di-*O*-methyl-deoxystreptamine (IV).

(2) Deoxystreptamine → *N*, *N'*-diacetyl-deoxystreptamine (V) → *N*,*N'*-diacetyl-*N*,*N'*-dimethyl-4, 5, 6-tri-*O*-methyldeoxystreptamine (VI) → *N*, *N'*-dimethyl-4, 5, 6-tri-*O*-methyl-deoxystreptamine (VII).

Scheme 1

*1 This constitutes Part XXV of a series entitled "Studies of Antibiotics and Related Substances," by Sumio Umezawa. A portion of this paper was presented at the 18th Annual Meeting of the Chemical Society of Japan, Osaka, April, 1965.

1) a) S. Umezawa, Y. Ito and S. Fukatsu, *J. Antibiotics*, **A11**, 120, 162 (1958); This Bulletin, **32**, 81 (1959); b) *J. Antibiotics*, **A12**, 187 (1959); S. Umezawa and Y. Ito, This Bulletin, **34**, 69 (1961).

2) S. Umezawa and T. Tsuchiya, *J. Antibiotics*, **A15**, 51 (1962).

3) M. Hichens and K. L. Rinehart, Jr., *J. Am. Chem. Soc.*, **85**, 1547 (1963).

4) R. E. Reeves, "Advances in Carbohydrate Chem.," Vol. 6, Academic Press, New York (1951), pp. 107—134.

5) S. Tatsuoka, S. Horii, K. L. Rinehart, Jr., and T. Nakabayashi, *J. Antibiotics*, **A17**, 88 (1964).

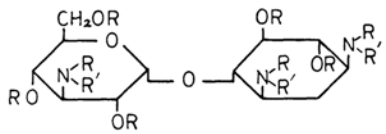
6) S. Umezawa, T. Tsuchiya and K. Tatsuta, This Bulletin, **39**, 1235 (1966).

*2 $\Delta[M]_{\text{TACu}} = ([\alpha]_{436}\ \text{TACu} - [\alpha]_{436}\ \text{water}) \times \frac{\text{Mol. wt.}}{100}$ -

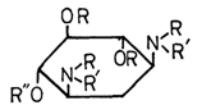
TABLE I.

	$[\alpha]_{589}^{15}$	$[\alpha]_{438}^{15}$	$\Delta[M]_{TACu}$	$\Delta[M]_{CuAm}$	$\Delta[M]_{Cupra\ B}$
IV·2HCl	+14	+30	+1100	+1090	+1110
VII·2HCl	+3	+6	+1	+10	−9
II	+80	+154	+40	+1590	+1420*

* Reported $\Delta[M]_{Cupra\ B}$ +1380 by Tatsuoka et al. (Ref. 5).

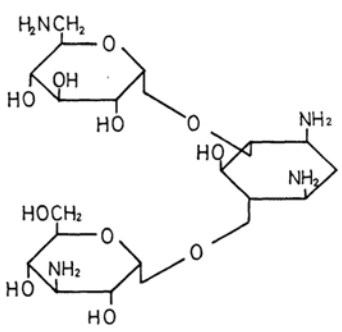


I : R=R'=H
II : R=H; R'=COCH$_3$
III : R=CH$_3$; R'=COCH$_3$



IV : R=CH$_3$; R'=R''=H
V : R=R''=H ; R'=COCH$_3$
VI : R=R''=CH$_3$; R'=COCH$_3$
VII R=R''=CH$_3$; R'=H

The three kinds of $\Delta[M]$ values for IV measured in TACu, CuAm and Cupra B were all approximately +1100, while those for VII were approximately zero, indicating that no complexing occurs between two methylamino groups of the deoxystreptamine moiety VII (Table I). These results, when subjected to the generalizations described in the preceding paper[6], led to the conclusion that the projected angle*3 between adjacent methylamino and hydroxyl groups is about +60° in the molecule of IV, that is, that IV is *N*, *N'* - dimethyl-4, 5-di-*O*-methyldeoxystreptamine; thus, kanamycin is deduced to be 4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-deoxystreptamine. This accords with the results obtained by the above-mentioned authors.[3,5] If IV were 5, 6-di-*O*-methyl isomer instead of the substance mentioned above, the $\Delta[M]_{TACu}$ value would be about −1000.



Kanamycin

The three $\Delta[M]$ values for II have also been measured to give an additional proof of the above structure assignment of kanamycin (Table I).

6-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-deoxystreptamine (I), which was first isolated by Maeda et al.,[7] was prepared by the hydrolysis of kanamycin with 3 N hydrochloric acid, heating them at about 100°C for one hour, followed by column chromatography using active carbon and Dowex 1×2; this procedure gave a high yield of I.

The acetylation of I has been effected by the use of acetic anhydride and barium hydroxide, thus giving II. The acetylation of deoxystreptamine briefly run in methanolic acetic anhydride gave V.

As to the methylation of II and V, a modified Purdie method was used; the methylation was effected in a sealed tube at 75—80°C. It has been found that the methylation of acetamino groups was performed as well as that of hydroxyl groups, affording III and VI respectively. This was proved by the determination of the infrared and NMR spectra; after the methylation, the absorption band at 1550 cm^{-1} (Amide II) observed in the infrared spectra of II and V disappeared in those of IV and VII respectively, while, in the NMR spectra of IV and VII, signals for *N*-methyl groups (~7.5τ) were clearly discerned (Fig. 1).

The hydrolysis of III and VI has been carried out in dilute hydrochloric acid, with the application of heat, giving IV and VII respectively.

Experimental

The preparation of a tetramminecopper(II) sulfate solution (TACu) and of cuprous chloride in 15 N aqueous ammonia (CuAm), and the determination of optical rotation were carried out as described in the preceding paper.[6] Paper chromatography was conducted by the descending technique on Toyo filter paper No. 50, and the locations of the substances on the papergrams were established by 0.25% ninhydrin in pyridine. Thin layer chromatography was carried out as described in the preceding paper, and the coloration of substances bearing amino groups was performed with concentrated sulfuric acid or 0.25% ninhydrin in pyridine.

6-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-deoxystreptamine (I).—A solution of a kanamycin base (10 g.) in 3 N hydrochloric acid (150 ml.) was heated in a boiling water bath for 1 hr. and then evaporated under reduced pressure. The resulting syrup was refluxed for 30 min. with dry methanol (500 ml.)

*3 See footnote of Ref. 6.

7) K. Maeda, M. Murase, H. Mawatari and H. Umezawa, *J. Antibiotics*, **A11**, 163 (1958).

containing dry hydrogen chloride (10 g.). By this procedure, aminosugars were converted to their glucosides; this was confirmed by paper chromatography. After the solution had been cooled, dry hydrogen chloride (100 g.) was gradually introduced, with the temperature kept at about −10°C, to precipitate a white solid. This solid was collected, washed with cold methanol and dried (8.5 g.). This was proved by paper chromatography to be composed of approximately equal amounts of I and deoxystreptamine, accompanied by 4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-deoxystreptamine (6AD) (minor product) and unchanged kanamycin. The filtrate contained deoxystreptamine, methyl 3-amino-3-deoxy-D-glucoside[8] and methyl 6-amino-6-deoxy-D-glucoside.[8] The solid (8.5 g.) was dissolved in a small quantity of water, placed on a column containing 100 g. of carbon (Wako Pure Chemical Industries, 70—150 mesh, activated at 120°C before use), and developed with water. After an initial portion (600 ml., ninhydrin negative) had been removed, the eluate was cut into 10 g. fractions; deoxystreptamine, 1, 6AD and kanamycin were then eluted in the fractions Nos. 4—18, 16—28, 27—50 and 45—63 respectively. The eluate of Nos. 16—28 was evaporated and dried (3.5 g.). An aqueous solution of the residue was passed through a column of Dowex 1×2 (OH form, 29×500 mm.) and eluted with water. After a small amount of deoxystreptamine had been eluted, the free base of I emerged in a 110—300 ml. portion. This was concentrated to a small volume and neutralized with hydrochloric acid; from the resulting solution, trihydrochloride of I was crystallized by adding methanol and ethanol. Yield, 2.8 g. (31%), m. p. 235—236°C (decomp.) (lit.,[7] 219—219.5°C (decomp.)), $[\alpha]_{589}^{13}$ +75.1°, $[\alpha]_{438}^{13}$ +140.6° (*c* 1.3, water) (lit.,[7] $[\alpha]_{D}^{28}$ +76.1°).

Found: C, 33.43; H, 6.66. Calcd. for $C_{12}H_{25}N_3O_7 \cdot 3HCl$: C, 33.31; H, 6.51%.

6-*O*-(3-Acetamido-3-deoxy-α-D-glucopyranosyl)-*N*, *N*'-diacetyl-deoxystreptamine (II). — Trihydrochloride (500 mg.) of I suspended in aqueous ethanol (7 : 3) was acetylated with acetic anhydride and barium hydroxide. After the solvent had been removed in vacuo, the residue was extracted with ethanol and then evaporated. An aqueous solution of the residue (708 mg.) was treated with Amberlite IRA-400 (OH form) and Amberlite IR-120 (H form) successively. After the solvent had been removed, the residue was crystallized from its methanol solution (1.5 ml.) by adding acetone (5 ml.); it gave 450 mg. of II, which was proved to be pure on thin layer chromatography (R_f, 0.29)*4 with a solvent system of *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1). A pure sample of II had a m. p. of 238—242°C (decomp.) (lit., m. p. 235—242°C,[5] 176—181°C[9] (decomp.)), $[\alpha]_{589}^{12}$ +80° (*c* 0.45, water) (lit.,[5] $[\alpha]_{D}^{27}$ +72°); IR spectrum (KBr disk): ~3400, 1645 (Amide I), 1557 (Amide II), 1415, 1375 cm^{-1}.

Found: C, 47.16; H, 6.71; N, 8.72. Calcd. for $C_{18}H_{31}N_3O_{10} \cdot 1/2H_2O$: C, 47.16; H, 7.04; N, 9.16%.

6-*O*-(3-Deoxy-2,4,6-tri-*O*-methyl-3-*N*-methylacetamido-α-D-glucopyranosyl)-*N*, *N*'-diacetyl-*N*, *N*'-dimethyl-4, 5-di-*O*-methyl-deoxystreptamine (III). —In a 10 ml.-glass-pressure tube coupled with a pressure gauge there were placed methyl iodide (2.5 g.) and a thoroughly-ground mixture of II (200 mg.) and silver oxide (2.0 g.); the mixture was then heated at 75°C for 3 hr. At the end of this period, the inner pressure reached to 3 atm. After cooling, the whole content was extracted twice with methanol, and the extract was evaporated to dryness. The methylation was repeated several times. During the course of the reaction, in the infrared absorption spectra of the resulting products, the absorption bands at ~3400 cm^{-1} (OH) and ~1550 cm^{-1} (Amide II) gradually faded and those at ~2920 cm^{-1} and 1450 cm^{-1} became clearer, while the absorption at ~1640 cm^{-1} (Amide I) remained unchanged. The final product thus obtained was dissolved in chloroform, and, after filtration, the solution was evaporated to dryness. An aqueous solution of the residue was treated with active charcoal and then evaporated to give a white solid, 187 mg.; m. p. 98—100°C, $[\alpha]_{D}^{18}$ +94° (*c* 1, water). On thin layer chromatography with dioxane, the product showed a single spot with an R_f of 0.2. IR spectrum (KBr disk): 2920, 2815, 1640, 1450, 1410, 1375 cm^{-1}.

Found: C, 55.42; H, 8.47; N, 7.51. Calcd. for $C_{26}H_{47}O_{10}N_3$: C, 55.59; H, 8.43; N, 7.48%.

***N*, *N*'-Dimethyl-4, 5-di-*O*-methyldeoxystreptamine (IV).** — A sample of III (400 mg.) was dissolved in 3 N hydrochloric acid (20 ml.) in a sealed tube and then heated in a boiling water bath for 6 hr. After evaporation in vacuo, the syrup was deacidified by co-evaporation with water. A solution of the residue in a solvent system of *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1) was chromatographed on a cellulose powder column (45×165 mm.) and eluted with the same solvent. Ninhydrin-active substances emerged in 100—120, 180—240 and 260—380 ml. portions. The last portion, which had no reducing property, was concentrated to a small volume and then again chromatographed through a Dowex 1×2 column (OH form, 8×320 mm.) with water. Ninhydrin-active fractions were collected and evaporated to dryness. The residue was dissolved in chloroform, and, after filtration, the solution was evaporated to dryness (140 mg.). The residue was dissolved in water, neutralized with hydrochloric acid, and evaporated to dryness to give a solid, which was then recrystallized from ethanol to give dihydrochloride of IV; yield, 120 mg.; m. p. 228—231°C (decomp.), $[\alpha]_{589}^{15}$ +13.6° (*c* 0.3 water); IR spectrum (KBr disk): 3360, 2940, 2825, 2740, 2440, 1585 (δNH_2^+), 1465, 1385, 1340 cm^{-1}.

Found: C, 41.00; H, 8.12; N, 9.37. Calcd. for $C_{10}H_{22}O_3N_2 \cdot 2HCl$: C, 41.24; H, 8.31; N, 9.62%.

The NMR spectrum of the base IV was determined at a frequency of 60 Mc.p.s. with a Varian A 60 spectrometer in deuteriochloroform, and also in the same solvent including a few drops of deuteroxide (Fig. 1). Tetramethylsilane was used as the internal reference.

***N*, *N*'-Diacetyldeoxystreptamine (V).**—A deoxystreptamine base (500 mg.) suspended in dry methanol (5 ml.) was vigorously stirred into acetic anhydride (1.3 ml.); the reaction was then continued for 3 hr.

8) H. Ogawa, T. Ito, S. Inoue and S. Kondo, ibid., **A11**, 70, 72 (1958).

*4 The free base I has an R_f value of 0.15 with the same solvent.

9) H. Ogawa, T. Ito, S. Kondo and S. Inoue, *J. Antibiotics*, **A11**, 169 (1958).

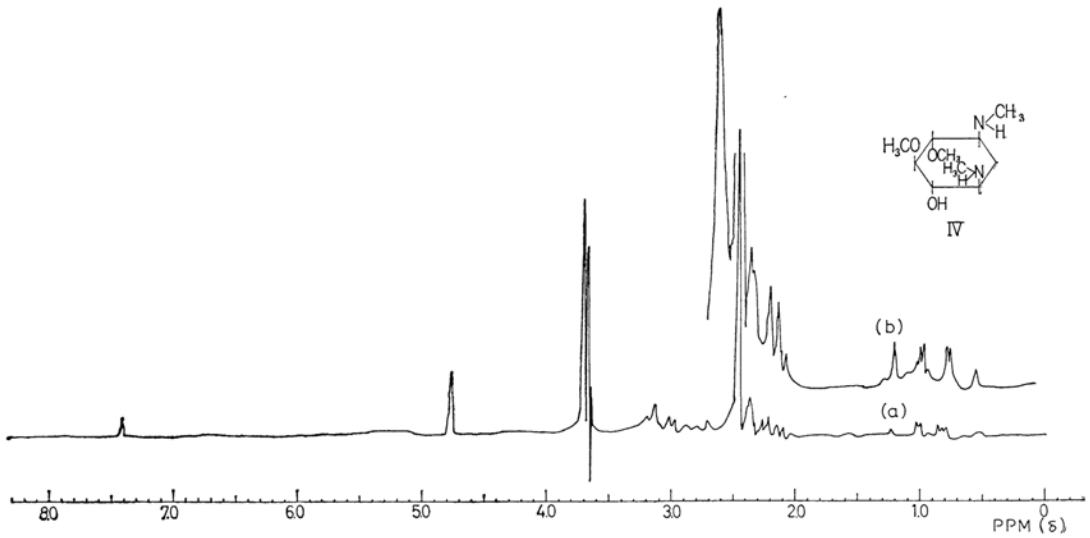


Fig. 1-A. The NMR spectra of *N*, *N'*-dimethyl-4, 5-di-*O*-methyldeoxystreptamine (IV); (a): in the mixture of $CDCl_3$ and a few drops of D_2O, (b): in $CDCl_3$.

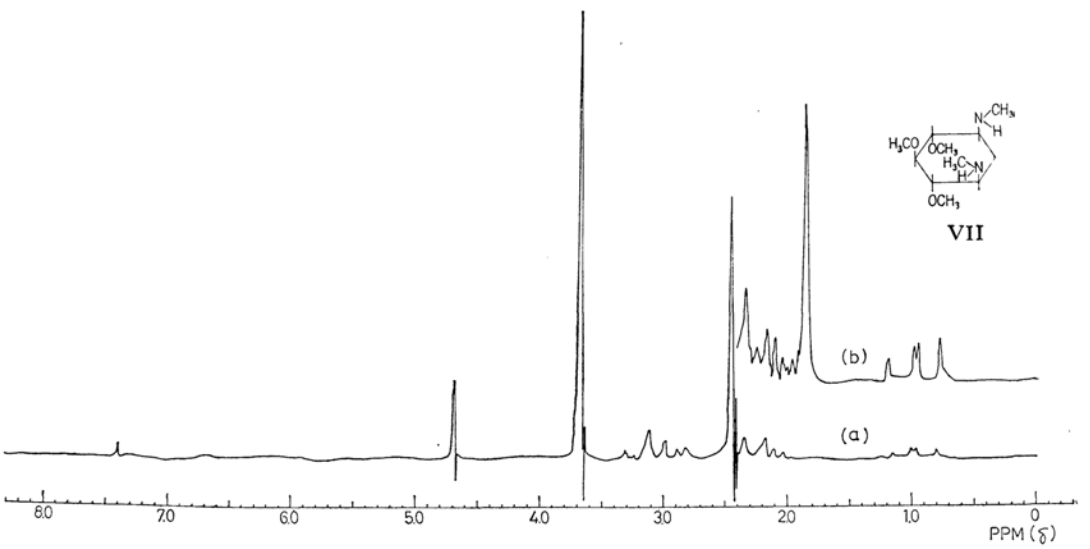


Fig. 1-B. The NMR spectra of *N*, *N'*-dimethyl-4, 5, 6-tri-*O*-methyldeoxystreptamine (VII); (a): in the mixture of $CDCl_3$ and a few drops of D_2O, (b): in $CDCl_3$.

The precipitate was filtered, washed with methanol, and then dissolved in water. To the solution, a small quantity of Amberlite IR-120 (H form) was added to remove the unchanged material; after filtration, the solution was concentrated to a small volume, and ethanol was added to cause crystallization. Yield, 570 mg.; m. p. 250°C (lit.,[10] 292—293°C); IR spectrum (KBr disk): 3450, 3340, 3300, 3120, 2900, 1650 (Amide I), 1580 (Amide II), 1445, 1417, 1385 cm^{-1}.

Found: C, 48.55; H, 7.28; N, 11.08. Calcd. for $C_{10}H_{18}O_5N_2$: C, 48.77; H, 7.37; N, 11.38%.

10) H. A. Luts, R. H. Sprague, M. Dickerson and L. C. Cheney, *J. Pharmaceutical Sciences*, **50**, 328 (1961).

***N*, *N'*-Diacetyl-*N*,*N'*-dimethyl-4, 5, 6-tri-*O*-methyldeoxystreptamine (VI).**—In a 20 ml.-glass-pressure tube there were placed methyl iodide (7.0 g.) and a thoroughly-ground mixture of V (200 mg.) and silver oxide (7.0 g.); the mixture was then heated at 85°C for 5 hr. After cooling, the reaction mixture was evaporated. The residue was extracted thrice with water, and the combined extracts were evaporated to dryness. The methylation was repeated thrice more. The change in infrared spectra during the repeated methylation was substantially the same as that concerning III. The product was dissolved in chloroform, and, after filtration, the solution was evaporated to give a white solid, 209 mg.; m. p. 207—208°C (decomp.); IR spectrum (KBr disk): 2920,

2820, 1640 (Amide I), 1445, 1410, 1380 cm^{-1}.

Found: C, 56.94; H, 8.92; N, 8.85. Calcd. for $C_{15}H_{28}O_5N_2$: C, 56.73; H, 8.71; N, 8.70%.

***N, N'*-Dimethyl-4, 5, 6-tri-*O*-methyl-deoxystreptamine (VII).**—A solution of VI (400 mg.) in 2 N hydrochloric acid (20 ml.) was heated in a boiling water bath for 5 hr. and then concentrated under reduced pressure. The residual syrup was deacidified by co-evaporation with water. A solution of the residue in a small quantity of a solvent mixture of *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1) was chromatographed on a cellulose powder column (45×250 mm.) with the same solvent, and each fraction (15 ml.) was tested by ninhydrin-coloration. Ninhydrin-active substances were eluted in Tubes Nos. 37—42 and Nos. 47—49. The former fractions were collected, evaporated, and dried. The residue (220 mg.) was dissolved in a small quantity of water and passed through a column of Dowex 1×2 (OH form, 8× 370 mm.) with water. The ninhydrin-active fractions were again collected and evaporated to dryness. The residue was dissolved in chloroform, and, after filtration, the solution was again evaporated to dryness. An aqueous solution of the residue was treated with active charcoal, neutralized with hydrochloric acid, and evaporated. Recrystallization from its methanol solution by adding acetone gave crystalline dihydrochloride of VII; yield, 155 mg.; m. p. 215—216°C (decomp.), $[\alpha]_{589}^{15}$ +3° (*c* 0.3, water). This has an R_{fIV}*5 value of 1.5 on paper chromatography with a solvent system of *t*-amylalcohol - pyridine - water - acetic acid (6 : 4 : 3 : 1). IR spectrum (KBr disk); 3380, 2940, 2820, 2720, 2450, 1610 (δNH_2^+), 1470, 1390 cm^{-1}.

Found: C, 43.19; H, 8.40; N, 8.97. Calcd. for $C_{11}H_{24}O_3N_2 \cdot 2HCl$: C, 43.28; H, 8.58; N, 9.18%.

The NMR spectrum of the base VII was determined as described in the procedure for IV (Fig. 1).

The authors are indebted to Mr. Saburo Nakada of this Laboratory for the microanalysis. This research was supported in part by a grant from the Microbial Chemistry Foundation, to which the authors' thanks are due.

*5 R_{fIV} is an R_f value relative to the R_f value of *N, N'*-dimethyl-4, 5-di-*O*-methyldeoxystreptamine (IV), the latter taken as 1.0.