Studies on Antibiotics and Related Substances. VII. The Structure of Kanamycin

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Kanamycin has been described by H. Umezawa and coworkers[1] as an antibiotic produced by a strain of *Streptomyces kanamyceticus*. It is low toxic and very active against a variety of Gram-positive and Gram-negative bacteria including streptomycin-resistant strain. Kanamycin was isolated in a pure state as a crystalline monosulfate which was converted into a crystalline free base[2,3]. Analysis and electrometric titrations of the salt and the free base indicated that the antibiotic is a tetra-acidic base of the composition $C_{18}H_{36}O_{11}N_4$. Acetylation of kanamycin with acetic anhydride in methanol gave tetra-*N*-acetyl kanamycin[3,4]. On the other hand, when kanamycin was acetylated with acetic anhydride and pyridine, undeca-acetylkanamycin was obtained[4].

Information about the degradation products of kanamycin was obtained by studying the products formed by acid hydrolysis[3,4]. From the hydrolyzate of kanamycin by 6 N hydrochloric acid, Maeda, et al.[4], isolated two kinds of reducing aminohexoses and diamino-trihydroxycyclohexane which was identical with deoxystreptamine (I) isolated from the vigorous hydrolysis of neomycin[5]. Moreover, Ogawa and Itō[6] obtained deoxystreptamine and two kinds of methylaminohexosides by methanolysis of kanamycin. Recently, we received an information from Hooper and others[7] that they identified the two aminohexose-moieties of kanamycin to be D-6-amino-6-deoxyglucose (pyranose form) (II) and 3-amino-3-deoxyaldohexose (pyranose form) (III).

Therefore, the hydrolytic cleavage of kanamycin could be represented by the following equation:

$$C_{18}H_{36}O_{11}N_4 + 2H_2O \rightarrow C_6H_{14}O_3N_2 + 2C_6H_{13}O_5N$$
(I) (II) and (III)

The present authors[8] recently reported in a short communication that the acid-hydrolysis of exhaustively methylated *N*-acetylkanamycin resulted in the isolation of mono-*O*-methyldeoxystreptamine which presents evidence to show that both of the two aminohexose moieties are directly joined to deoxystreptamine. The situation is different from streptomycin in which streptidine is linked with streptobiosamine, or from neomycin in which deoxystreptamine is linked with neobiosamine.

The present paper deals with the sites of glucosidic linkages occurring between the above-mentioned three moieties in detail.

The tetra-*N*-acetylkanamycin prepared by the method of Cron and others[3] was methylated with dimethyl sulfate and sodium hydroxide by the method of West and Holden[9] to yield a crude methylated product, which was chromatographed through a cellulose powder column to yield a colorless gum. The acetyl determination showed that the methylated product is partially de-*N*-acetylated. When the methylated product was acetylated again to make sure, the tetra-*N*-acetyl-hepta-*O*-methylkanamycin was obtained. It was further ascertained by infrared spectrum that no free hydroxyl group is contained in the methylated product.

The methylated product was hydrolyzed with 6 N hydrochloric acid. Paper chromatography studies of the hydrolyzate showed three ninhydrin positive spots of

1) H. Umezawa, M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and T. Takeuchi, *J. Antibiotics, Series A*, **10**, (5), 181 (1957).
2) K. Maeda, M. Ueda, K. Yagishita, S. Kawaji, S. Kondo, M. Murase, T. Takeuchi, Y. Okami and H. Umezawa, ibid., 228 (1957).
3) M. J. Cron, D. L. Johnson, F. M. Palermiti, Y. Perron, H. D. Taylor, D. F. Whitehead and I. R. Hooper, *J. Am. Chem. Soc.*, **80**, 752 (1958).
4) K. Maeda, M. Murase, H. Mawatari and H. Umezawa, *J. Antibiotics, Series A*, **11**, (2), 73 (1958).
5) F. A. Kuehl, M. N. Bishop, K. Folkers, L. H. Sommer, N. S. Marans, G. M. Goldberg, J. Rockett and P. P. Pioch, *J. Am. Chem. Soc.*, **73**, 881 (1951).
6) H. Ogawa and T. Itō, *J. Antibiotics, Series A*, **10**, (6), 267 (1957); ibid., **11**, (2), 70, 72 (1958).
7) M. J. Cron, O. B. Farding, D. L. Johnson, H. Schmitz, D. F. Whitehead, I. R. Hooper and R. V. Lemieux, *J. Am. Chem. Soc.*, **80**, 2342, 4741 (1958).
8) S. Umezawa, Y. Itō and S. Fukatsu, *J. Antibiotics, Series A*, **11** (5), 120 (1958).
9) E. S. West and R. F. Holden, *J. Am. Chem. Soc.*, **56**, 930 (1934).

R_f 0.08, 0.32 and 0.41 with a *n*-butanol-ethanol-water mixture. The hydrolyzate was evaporated to dryness, taken up in methanol and treated with ether to yield an amorphous precipitate, which consisted of the product of R_f 0.08, containing a small amount of the product of R_f 0.32. The precipitate was chromatographed through a cellulose powder column to yield the colorless, crystalline hydrochloride of mono-*O*-methyldeoxystreptamine (IV), which was optically inactive and, upon oxidation with periodate, consumed two moles of periodate without the formation of formic acid.

The hydrochloride was acetylated with acetic anhydride in pyridine to yield the colorless prisms of the diacetate (V) of mono-*O*-methyl-di-*N*-acetyldeoxystreptamine.

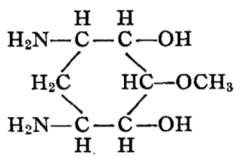
Finally, the diacetate was subjected to de-*O*-acetylation with ammonia in absolute methanol solution at 0°C to yield the colorless prisms of mono-*O*-methyl-di-*N*-acetyldeoxystreptamine (VI). This consumed no periodate and was also optically inactive.

The compounds (IV, VI) with the *O*-methyl group in the 5-position should be optically inactive while the compounds with the *O*-methyl group in the 4- or 6-position should be optically active. The di-*N*-acetyl compound (VI) with the *O*-methyl group in the 5-position should consume no periodate while the corresponding compound with the *O*-methyl group in the 4- or 6-position should consume one mole of periodate. The compound IV with the *O*-methyl group in the 5-position should be oxidized with two moles of periodate without the formation of formic acid while the corresponding compound with the *O*-methyl group in the 4- or 6- position should consume two moles of periodate with the formation of one mole of formic acid.

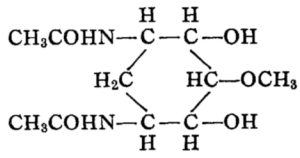
Therefore, the experimental evidences presented above have led the authors to conclude that the mono-*O*-methyl-deoxystreptamine (IV) is 1,3-diamino-4,6-dihydroxy-5-methoxycyclohexane and, considered with the previous findings, permit the assignment of structure VII to kanamycin.

Experimental

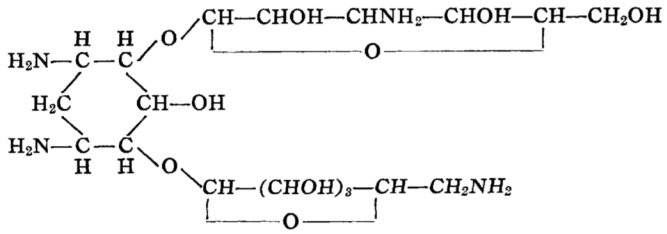
Methylation of *N*-Acetylkanamycin.—To a vigorously stirred solution of tetra-*N*-acetylkanamycin (4.0 g.) in water (8 cc.) was added a solution of dimethyl sulfate (52 g.) in carbon tetrachloride (40 cc.). During this and the subsequent addition of alkali the temperature of the reaction was kept at 50~55°C. A solution of sodium hydroxide (108 g.) in water (100 cc.) was then added dropwise over a one-hour period, with vigorous stirring. The temperature was then raised slowly to 70~75°C and dimethyl sulfate (103 g.) was added dropwise over a 30-minute period. Again, a solution of sodium hydroxide (36 g.) in water (30 cc.) was added and then dimethyl sulfate (52 g.) was added gradually in a 15-minute period. After this addition the water-bath was heated to boiling and kept at this temperature for 30 min. The temperature was then lowered to about 50°C and the mixture was stirred vigorously with chloroform (150 cc.) for 15 min. The mixture was filtered and the sodium sulfate precipitate was washed with chloroform. The aqueous portion of the filtrate was re-extracted with chloroform as described above. The chloroform extracts were combined and dried with sodium sulfate. Evaporation of the solvent in vacuo left an amber



(IV)



(VI)



(VII)
Kanamycin

gum (3.22 g.) soluble in methanol, ethanol, chloroform and water. A sample was dried in vacuo at 60~70°C. The determination of methoxyl content gave 27.44% OCH_3.

The crude product (11.17 g.) was dissolved in a solution (30 cc.) of *n*-butanol-ligroin-water (38 : 60 : 2) and chromatographed on a column (2.5×40 cm.) of cellulose powder washed with the same solvent. Sixteen fractions of eluate with the same solvent were limited to 40 cc. each, evaporated in vacuo to dryness and weighed. Fractions 7-14 were combined to yield 7.0 g. of exhaustively methylated *N*-acetyl-kanamycin, m. p. 80°C (sintered at about 65°C). The determinations of methoxyl and acetyl content gave 28.35% OCH_3 and 12.25% $COCH_3$.

It was ascertained by infrared spectrum that no free hydroxyl group is contained in the methylated product.

A portion (2.5 g.) of the methylated product of tetra-*N*-acetylkanamycin was dissolved in absolute methanol (22.5 cc.) and to the solution acetic anhydride (4.3 cc.) was added. After being allowed to stand overnight, the acetylation mixture was filtered and evaporated to dryness. The residue was dissolved in water (20 cc.) and extracted thrice with chloroform (20 cc.). The chloroform extracts were combined and dried with sodium sulfate. Evaporation of the solvent left a colorless gum which resisted attempts to crystallize it (1.17 g.).

Anal. Found: OCH_3, 27.5; $COCH_3$, 21.6. Calcd. for $C_{18}H_{21}O_4(OCH_3)_7(NHCOCH_3)_4$: OCH_3, 28.9; $COCH_3$, 22.9. Calcd. for $C_{18}H_{22}O_5(OCH_3)_6(NHCOCH_3)_4$: OCH_3, 25.3; $COCH_3$, 23.4%.

Isolation of Mono-*O*-methyldeoxystreptamine Hydrochloride (IV) from Hydrolyzed Methylated *N*-acetylkanamycin.—Exhaustively methylated *N*-acetyl-kanamycin (7.0 g.) was refluxed with 6 N hydrochloric acid (100 cc.) for 1.5 hr. The reaction mixture was evaporated to dryness at 60°C in vacuo. The residue was dissolved in water (100 cc.) and evaporated again to dryness. The evaporation to remove hydrogen chloride was repeated thrice. The residue was dissolved in water (100 cc.) and the solution was decolorized with active charcoal (1.0 g.).

Paper chromatography of the hydrolyzate using the top-layer of *n*-butanol-ethanol-water (4 : 1 : 5) gave 3 zones with ninhydrin. The R_f values were 0.08 (brown), 0.32 (orange) and 0.41 (violet), respectively.

The decolorized solution was evaporated at 60°C in vacuo to dryness and dried in a desiccator overnight. The amber residue was dissolved in methanol (20 cc.) and the solution was diluted with ether (50 cc.) to yield a precipitate (1.90 g.). Paper chromatography of the precipitate using the above-mentioned solvent showed the main spot with R_f 0.08 (brown) and a trace with R_f 0.32 (orange).

The precipitate (1.90 g.) was dissolved in the top-layer (10 cc.) of *n*-butanol-ethanol-water (4 : 1 : 5) and chromatographed on a column (1.5×50 cm.) of cellulose powder (18 g.) washed with the same solvent. Twenty fractions of eluate with the same solvent were limited to 20 cc. each, evaporated to dryness in vacuo and weighed. Fractions 6—9 were combined to yield the crude hydrochloride of mono-*O*-methyldeoxystreptamine (1.65 g.).

Three recrystallizations from methanol-ethanol gave colorless needles (350 mg.) of the hydrochloride of 1, 3-diamino-4, 6-dihydroxy-5-methoxycyclohexane (IV), m. p. 207~209°C (decomp.), which was optically inactive.

Anal. Found: C, 34.03; H, 7.17; N, 10.94; OCH_3, 11.65. Calcd. for $C_6H_7(OH)_2(NH_2)_2(OCH_3)\cdot 2HCl$: C, 33.73; H, 7.23; N, 11.24; OCH_3, 12.45%.

Periodate Oxidation. — The accurately weighed sample (20.1 mg.) of 1, 3-diamino-4, 6-dihydroxy-5-methoxycyclohexane hydrochloride was dissolved in 0.023 N sodium periodate solution (20 cc.) and 2 cc. aliquots were titrated with 0.1 N sodium arsenite at the intervals listed in Table I, according to the procedure of Fleury and Lange[10]. Two moles of periodate was consumed without the formation of formic acid.

TABLE I
PERIODATE OXIDATION*

	Time (hr.)					
	0.5	1	2	3	4	6
1, 3-Diamino-4, 6-dihydroxy 5-methoxy-cyclohexane·2HCl	1.91	1.88	—	1.98	—	1.95
1, 3-Diacetamido-4, 6-dihydroxy-5-methoxy-cyclohexane	0.18	0.25	0.14	—	0.23	—

* Expressed in terms of moles of periodate consumed per mole of substance.

1, 3-Diacetamido-4, 6-diacetoxy-5-methoxycyclohexane (V). — The crude hydrochloride (1.2 g.) of 1, 3-diamino-4, 6-dihydroxy-5-methoxycyclohexane was acetylated with acetic anhydride-pyridine (18 cc. : 36 cc.) at 27°C for 24 hr. The acetylation mixture was concentrated to dryness at 40°C in vacuo; the residue was dissolved in water (20 cc.) and decolorized with active charcoal (0.5 g.). The filtrate was again evaporated to dryness in vacuo and dried in a desiccator overnight. The residue was washed with ether (30 cc.), and taken up in chloroform (20 cc.); a small quantity of insoluble part was filtered off and the filtrate was dried with sodium sulfate. Evaporation of the solvent was followed by extraction with hot ethyl acetate. Crystallization from the extract yielded colorless prisms (215 mg.), m. p. 220°C (decomp. sintered at about 170°C).

Anal. Found: C, 52.35; H, 6.65; N, 8.18; OCH_3, 9.22. Calcd. for $C_6H_7(OCH_3)(OCOCH_3)_2\cdot(NHCOCH_3)_2$: C, 52.32; H, 6.98; N, 8.14; OCH_3, 9.01%.

10) P. F. Fleury and J. Lange, *J. pharm. chim.*, [8] **17**, 107, 196 (1933).

The mother liquor was concentrated and allowed to stand at room temperature to yield the second crop of crystals (350 mg.).

1,3-Diacetamido-4,6-dihydroxy-5-methoxycyclohexane (VI). — A solution of 1,3-diacetamido-4,6-diacetoxy-5-methoxycyclohexane (460 mg.) in absolute methanol (130 cc.) was treated with a stream of dry ammonia gas for 1 hr. After being allowed to stand 3 hr. at room temperature, the mixture was evaporated at about 50°C in vacuo to dryness. The residue was dissolved in absolute methanol (50 cc.) and evaporated again to dryness in vacuo. The residue was taken up in absolute ethanol (30 cc.) and the solution was filtered to remove a small quantity of insoluble part. When the filtrate was concentrated and cooled, colorless needles crystallized out. Recrystallization from absolute ethanol yielded 130 mg. of 1,3-diacetamido-4,6-dihydroxy-5-methoxycyclohexane. The product was optically inactive.

Anal. Found: C, 50.94; H, 7.47; N, 10.64; OCH_3, 11.32; *N*-acetyl, 32.99, 33.87. Calcd. for $C_6H_7(OH)_2(OCH_3)(NHCOCH_3)_2$: C, 50.77; H, 7.69; N, 10.77; OCH_3, 11.9. *N*-acetyl, 33.08%.

Periodate Oxidation. — An accurately weighed sample (25.3 mg.) of 1,3-diacetamido-4,6-dihydroxy-5-methoxycyclohexane was dissolved in 0.0287 N sodium periodate (20 cc.) and 2 cc. aliquots were titrated with 0.1 N sodium arsenite at the intervals listed in Table I.

Summary

Evidence is presented to show that both the two aminohexose moieties in kanamycin are directly joined to deoxystreptamine through hydroxyl groups by glucosidic linkages. Proof for the sites of attachment of the two aminohexose moieties rests on the periodate oxidation of the optically inactive 1,3-diamino-4,6-dihydroxy-5-methoxycyclohexane and its *N*-acetyl derivative which were obtained by the degradation of exhaustively methylated *N*-acetylkanamycin by acid hydrolysis followed by acetylation. Kanamycin is therefore represented by formula VII.

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