THE SYNTHESIS OF KANAMYCIN-6″-URONIC ACID, ITS ESTER AND AMIDE

TAKEO KOBAYASHI, TSUTOMU TSUCHIYA, KUNIAKI TATSUTA and SUMIO UMEZAWA

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

(Received for publication March 19, 1970)

Penta-O-acetyl-tetra-N-ethoxycarbonyl-6″-O-tritylkanamycin* was prepared, and, after detritylation, oxidized with potassium permanganate in acetic acid. The oxidation product was esterified with diazomethane to give methyl penta-O-acetyl-tetra-N-ethoxycarbonylkanamycin-6″-uronate. Hydrolysis of the product with barium hydroxide followed by column chromatography and esterification with methanolic hydrogen chloride afforded methyl kanamycin-6″-uronate. Hydrolysis of the ester with dilute hydrochloric acid afforded kanamycin-6″-uronic acid. The ester was converted into its amide. An alternative oxidation of tetra-N-benzyloxycarbonylkanamycin using platinum and oxygen also led to the same uronic acid. The antibiotic spectra of the uronic acid and its ester and amide are described.

In earlier papers,[1,2] we have reported the syntheses, antibacterial spectra, and toxicity of a series of kanamycin derivatives in which the primary hydroxyl group in the 3-amino-3-deoxy-D-glucose moiety was replaced with several kinds of groups. The present paper is concerned with the extension of this work and reports the introduction of a carboxyl group, that generally lowers the toxicity of drugs, into kanamycin.

Tetra-N-ethoxycarbonylkanamycin (I) was prepared and selectively tritylated with trityl chloride in dry pyridine at 50°C for 48 hours to give tetra-N-ethoxycarbonyl-6″-O-tritylkanamycin (II) in a 77 % yield. Acetylation of II with acetic anhydride in pyridine at 50°C for 24 hours led to the formation of penta-O-acetyl-tetra-N-ethoxycarbonyl-6″-O-tritylkanamycin (III) in a 91 % yield. It has been found that the hydroxyl group at C-5 of 2-deoxystreptamine moiety remained unsubstituted in this acetylation.

Detritylation of III by treatment with 80 % aqueous acetic acid at 40°C afforded penta-O-acetyl-tetra-N-ethoxycarbonylkanamycin (IV) in a 95 % yield.

Oxidation of IV with potassium permanganate at room temperature for 6 days afforded a crude oxidation product, however, no analytically pure oxidation product could be isolated by usual chromatography. Therefore, the crude product was processed in the following manner.

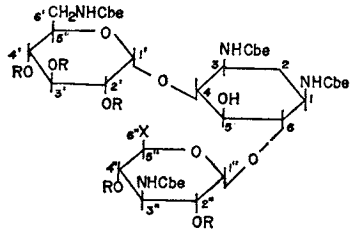
The crude oxidation product was esterified with diazomethane to give methyl

* The kanamycin structure is numbered as shown in the formula of chart 1; 6-amino-6-deoxyglucose ring is numbered with prime and 3-amino-3-deoxyglucose ring with double primes.

penta-O-acetyl-tetra-N-ethoxycarbonylkanamycin-6″-uronate (V), m. p. 246~248°C, $[\alpha]_D^{15}$ +65° (*c* 1, chloroform), in a 41 % yield.

The masked uronate (V) was refluxed with 1 N barium hydroxide for 5 hours to remove the protecting groups and the product was purified by column chromatography with Dowex 1×2 resin. However, since the paper chromatography of the product showed the presence of two materials with Rf_{KM}* 0.34 (main) and 0.73 (with a solvent system: *n*-butanol - pyridine - water - acetic acid; 6 : 4 : 3 : 1) and efforts to separate the two components by usual column chromatograpy with elution procedure were unsuccessful, the mixture was separated by column chromatography with extrusion procedure; the individual bands of the chromatogram of the cellulose powder were pushed out from the tube and the components in each band were eluted with 1 N hydrochloric acid. The product of Rf_{KM} 0.34 (VI), which showed antibacterial activity against *Bacillus subtilis* on a bioautogram, was further purified with Dowex 1×2 resin to give a powder of the desired kanamycin-6″-uronic acid in a yield of about 26 %. Since the product still included a slight amount of inorganic salts, it was converted to the ester by treatment with methanolic hydrogen chloride to give the tetrahydrochloride of methyl kanamycin-6″-uronate (VII), m. p. about 228°C (decomp.), $[\alpha]_D^{15}$ +84° (*c* 1, water) in a 21 % overall yield from V.

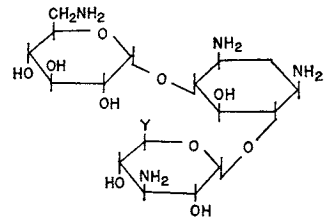
Chart 1.



	R	X
I:	H	CH_2OH
II:	H	CH_2OTr
III:	Ac	CH_2OTr
IV:	Ac	CH_2OH
V:	Ac	$COOCH_3$

Cbe: COOEt

Chart 2.



	Y
Kanamycin:	CH_2OH
VI:	COOH
VII:	$COOCH_3$
VIII:	$CONH_2$

Table 1. Antibacterial spectra of kanamycin-6″-uronic acid (VI), its ester (VII) and amide (VIII)

Test organisms	M. I. C.* (mcg/ml)			
	VI·3HCl	VII·4HCl	VIII·4HCl	Kanamycin·4HCl
Staphylococcus aureus FDA 209P	25	25	100	3.12
Ditto (kanamycin resistant)	>100	>100	>100	>100
Bacillus subtilis PCI 219	3.12	3.12	12.5	0.78
Escherichia coli NIHJ	12.5	12.5	50	3.12
Escherichia coli K-12 CS-2	12.5	6.25	100	6.25
Klebsiella pneumoniae PCI 602	12.5	12.5	50	3.12
Pseudomonas aeruginosa 87	50	100	>100	25
****Mycobacterium smegmatis* ATCC 607	12.5	25	25	1.56
****Mycobacterium phlei*	12.5	25	25	1.56

* Nutrient agar 37℃, 18-hour culture
** Nutrient agar 37℃, 42-hour culture

* Rf-value relative to kanamycin, which is taken as 1.

Finally, hydrolysis of **VII** with 0.1 N hydrochloric acid at room temperature afforded a crystalline trihydrochloride of kanamycin-6″-uronic acid (**VI**), m. p. about 210°C (decomp.), $[\alpha]_D^{15}$ +92° (*c* 1, water), in a 89 % yield.

Treatment of the hydrochloride of the uronate (**VII**) with methanolic ammonia afforded the tetrahydrochloride of kanamycin-6″-uronamide (**VIII**), m. p. about 227°C (decomp.), $[\alpha]_D^{15}$ +95.5° (*c* 1, water) in a 92 % yield.

The minor product of Rf_{KM} 0.73 showed no antibacterial activity against *Bacillus subtilis* on a bioautogram, and was not studied further.

As an alternative method of oxidation, catalytic oxidation[8] of tetra-N-benzyloxycarbonylkanamycin[1] (**IX**) with platinum and oxygen was also carried out at 70~80°C, pH 7~8 for about 30 hours. The catalyst was replaced with fresh catalyst several times. Hydrogenolysis of the product to remove the protecting groups with palladium black in dioxane-water at pH 3~4 gave a crude uronic acid derivative of kanamycin. We again encountered trouble in the purification of the product. The product was purified by preparative paper chromatography to give the final product (**VI**) in a 9 % overall yield from **IX**. The identity of **VI** obtained by the two methods was confirmed by the physical data, infrared spectra and antibacterial spectra.

It has been found that the introduction of a carboxyl group into kanamycin considerably lowers the toxicity (LD_{50} 550 mg/kg mice, intravenously) and antibacterial activity *in vitro* as shown in Table 1. The ester derivative (**VII**) is similarly active to the carboxy derivative. The carboxamide derivative (**VIII**) was found to be less active than the carboxy and ester derivatives.

Experimental

Thin-layer chromatography (TLC) was performed by the use of silica gel (Silica-Rider, Daiichi Pure Chemicals Co., Japan).

Tetra-N-ethoxycarbonylkanamycin (I): To a suspension of kanamycin base (3.0 g) and anhydrous sodium carbonate (2.0 g) in a 50 % aqueous acetone (60 ml), carboethoxy chloride (2.31 ml) was added all at once with vigorous stirring. An almost clear solution was soon formed, and it shortly began to deposit precipitates. After having been set aside at room temperature overnight, water (40 ml) was added with shaking and the mixture was centrifuged. The product was then suspended in water and again centrifuged. The procedure was repeated several times to remove inorganic salts. The product (3.82 g, 80 %) was chromatographically homogeneous (Rf 0.37, TLC with methanol - benzene 2 : 5). It was purified by treatment with methanol-benzene; m. p. 302~303°C (decomp.), $[\alpha]_D^{22}$ +96° (*c* 1, DMF); IR spectrum (KBr disk): 1700 (NHCOOEt), 1535 cm^{-1} (NHCOO). Found: C 46.41, H 6.82, N. 7.04. Calcd. for $C_{30}H_{52}O_{19}N_4$: C 46.63, H 6.74, N 7.25 %.

Tetra-N-ethoxycarbonyl-6″-O-tritylkanamycin (II): To a solution of well dried I (10 g) in dry pyridine (200 ml), trityl chloride (7.27 g) was added and the solution was heated for 48 hours at 50°C. Evaporation of the solution followed by coevaporation with toluene several times gave a residue, which was dissolved in chloroform. The solution was washed with water, dried over sodium sulfate and evaporated to give a solid (14.6 g). This was dissolved in acetone - ethyl acetate (2 : 1) and passed through a silica gel column (100 g) with the same solvent. After trityl carbinol had been eluted, the desired compound II began to appear; 10.1 g (77 %); Rf 0.60 (TLC with acetone-ethyl acetate 2 : 1); m. p. 142~143°C; $[\alpha]_D^{15}$ +45° (*c* 1, $CHCl_3$); IR spectrum (KBr disk): 1700, 1535, 700 cm^{-1} (trityl). Found: C 57.77, H 6.84, N 5.55. Calc. for $C_{49}H_{66}O_{19}N_4$: C 57.98, H 6.55, N 5.52 %.

Penta-O-acetyl-tetra-N-ethoxycarbonyl-6″-O-tritylkanamycin (III): To a solution of II (6.77 g) in dry pyridine (136 ml), acetic anhydride (6.8 ml) was added and the solution was heated at 50°C for 24 hours. When the reaction was monitored by TLC (ethyl acetate), starting material (R*f* about 0) soon disappeared and several intermediate products (R*f*<0.7) formed by partial acetylation, gradually converged to one product (R*f* 0.74). The solution was poured into ice water and the resulting mass was filtered and washed throughly with water to give a crystalline solid (7.4 g, 91 %); m. p. 143°C; $[\alpha]_D^{15}$ +86° (*c* 1, $CHCl_3$); IR spectrum (KBr disk): ~3450 (OH), 1755 (AcO), 1710 (NHCOOEt), 705 cm^{-1}. Found: C 57.82, H 6.30, N 4.71. Calcd. for $C_{59}H_{76}O_{24}N_4$: C 57.84, H 6.25, N 4.57 %.

Penta-O-acetyl-tetra-N-ethoxycarbonylkanamycin (IV): A solution of III (3.55 g) in 80 % aqueous acetic acid (40 ml) was heated at 40°C. After about 40 minutes, the crystals of tritylcarbinol began to deposit and the hydrolysis was completed after 1.5 hour (by TLC of the solution with ethyl acetate, only one product of R*f* 0.41 was shown). Tritylcarbinol was removed by extration with benzene (10 ml×4) and the residual acidic solution was coevaporated with toluene *in vacuo* several times to give a colorless solid (2.7 g, 95 %); m. p. 244~246°C; $[\alpha]_D^{15}$ +74° (*c* 1, $CHCl_3$); IR spectrum (KBr disk): ~3400, 1750, 1700, 1540 cm^{-1}. Found: C 48.95, H 6.47, N 5.72. Calcd. for $C_{40}H_{62}O_{24}N_4$: C 48.88, H 6.36, N 5.70 %.

Methyl penta-O-acetyl-tetra-N-ethoxycarbonylkanamycin-6″-uronate (V): To a solution of IV (2.45 g) in acetic acid (50 ml), potassium permanganate (2.50 g) was added and the mixture was allowed to stand at room temperature for 6 days. Zinc dust was added until excess permanganate was exhausted. The reaction mixture was then evaporated *in vacuo* and the residue was extracted with chloroform. The chloroform solution was washed with 1 N hydrochloric acid and water successively, dried over sodium sulfate and evaporated *in vacuo* to give a colorless solid (1.69 g), which was purified by passing through a silica gel (100 g) column with ethyl acetate; yield 1.15 g.

Since chromatography did not yield an analytically pure product, it was purified through esterification as follows: To a solution of the product (4.73 g) in dioxane (30 ml), diazomethane in ether was added and the reaction was monitored by TLC (ethyl acetate); After the starting material (R*f* 0~0.15 with tailing) soon disappeared, the ester showing a clear-cut singlet (R*f* 0.68) appeared. Evaporation *in vacuo* gave a solid, which was purified with dioxane-ether; 4.23 g (41 % overall yield from IV); m. p. 246~248°C; $[\alpha]_D^{15}$ +65° (*c* 1, $CHCl_3$); IR spectrum (KBr disk): ~3550, ~1755, ~1740 cm^{-1}. Found: C 48.97, H 6.24, N 5.49. Calcd. for $C_{41}H_{62}O_{25}N_4$: C 48.71, H 6.18, N 5.54 %.

Methyl kanamycin-6″-uronate (VII) tetrahydrochloride: A suspension of V (5.0 g) in 1 N barium hydroxide solution (300 ml) was heated at 100°C for 3 hours, when the reaction mixture became to show strong ninhydrin color reaction. After carbon dioxide had been introduced, the precipitate was removed by centrifuge and the supernatant layer was concentrated. The concentrate was then charged on a column of Dowex 1×2 (OH form, 3.5×33 cm) and washed thoroughly with water; this procedure was carried out to remove any nonacidic impurities including kanamycin if present. The washing solvent was then changed to 0.1 N hydrochloric acid to eluate kanamycin-uronic acid (1.52 g). However, paperchromatography of the product with *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1) (Solvent A) indicated that the product still contained two components of Rf_{KM} 0.34 (main) and 0.73 (approximately one-tenth of the main product in the strength of ninhydrin coloration). Since efforts to separate the two components by usual elution procedure were unsuccessful, the separation was performed by pushing out the individual bands of the chromatogram from the tube as follows: the product (696 mg) was charged on a column (3.5×26 cm) of cellulose powder (Whatman CF-11, 75 g) and developed with Solvent A (2 liters) (the solvent system was found to be excellent for separation of the two products, although it developed the chromatogram very slowly). The chromatogram was pushed out and divided into ten parts, each of which was extracted with 0.1 N

hydrochloric acid. The fractions of Rf_{KM} 0.34 and 0.73 were collected, evaporated and further purified by Dowex 1×2 (OH form) by a procedure similar to that described above. The substance (**VI**, Rf 0.34) weighed 360 mg (~26 %) and the substance of Rf 0.73, 45 mg, the latter having no antibacterial activity. The former substance (322 mg) was suspended in 1 N methanolic hydrogen chloride (20 ml) and allowed to stand for 4 hours. Concentration *in vacuo* followed by coevaporation with benzene gave a solid, which was purified by aqueous methanol-acetone; 274 mg (**VII** tetrahydrochloride, 21 % based on **V**); Rf_{KM} 1.53 (paperchromatography with Solvent A); m. p. ~228°C (decomp.); $[\alpha]_D^{15}$ +84° (c 1, water); IR spectrum (KBr disk): 1735 cm^{-1}; NMR (in D_2O): τ: 6.12 (3-H. s., $COOCH_3$), 4.8 and 4.5 (each one appeared as 1-H. d., J~3.5 Hz, anomeric hydrogens). Found: C 34.43, H 5.88, N 8.44, Cl 21.20. Calcd. for $C_{19}H_{36}O_{12}N_4 \cdot 4HCl$: C 34.66, H 6.12, N 8.51, Cl 21.54 %.

Kanamycin-6″-uronic acid (**VI**) trihydrochloride: A solution of **VII** (200 mg) in 0.1 N hydrochloric acid was allowed to stand at room temperature for 5 hours, and the solution was evaporated *in vacuo*. Addition of acetone to the residue gave a solid (164 mg, 89 %); Rf_{KM} 0.34 (Solvent A); m. p. ~210°C (decomp.); $[\alpha]_D^{15}$ +92° (c 1, water). Found: C 35.71, H 6.50, N 8.74, Cl 17.12. Calcd. for $C_{18}H_{34}O_{12}N_4 \cdot 3HCl$: C 35.56, H 6.14, N 9.21, Cl 17.50 %.

Kanamycin-6″-uronamide (**VIII**) tetrahydrochloride: To a solution of **VII** (52.8 mg) in methanol (2 ml), saturated ammoniacal methanol (0.2 ml) was added and the solution was allowed to stand overnight. Evaporation *in vacuo* gave a solid, which was dissolved in a small amount of 0.1 N hydrochloric acid. Addition of a mixture of methanol-ethanol gave the tetrahydrochloride of kanamycin-6″-uronamide (**VIII**); 47.4 mg (92 %); Rf_{KM} 0.56 (Solvent A); m. p. ~227°C (decomp.); $[\alpha]_D^{15}$ +95.5° (c 1, water); IR spectrum (KBr disk): 1680 cm^{-1} (amide). Found: C 33.70, H 6.16, N 10.93, Cl 21.76. Calcd. for $C_{18}H_{35}O_{11}N_5 \cdot 4HCl$: C 33.61, H 6.11, N 10.89, Cl 22.04 %.

Preparation of **VI** by a catalytic oxidation method: To a solution of tetra-N-benzyloxycarbonylkanamycin[1] (1.0 g) in aqueous-dioxane (1 : 4, 75 ml), a small volume of 5 % aqueous sodium hydrogen carbonate was added. Adams platinum oxide (400 mg), which was activated with hydrogen just before use, was added and oxygen was bubbled through the suspension at 80~85°C in a speed at 2 liters/hour. Every 15 hours, the catalyst was replaced and the oxidation was continued for a total of 45 hours. Additional portions of 5 % aqueous sodium hydrogen carbonate were added from time to time to maintain the pH of the suspension between 7.5~8 (total 4 ml). At the end of the reaction, no further change in pH was observed. The suspension was filtered and the filtrate was evaporated to give a pale brown solid, which was ground with a small volume of water, filtered, and washed throughly with water to give a pale yellow solid (436 mg).

The solid (400 mg) was dissolved in aqueous-dioxane (1 : 4, 15 ml) and the solution was adjusted to pH 2 by the addition of 0.1 N hydrochloric acid. The solution was hydrogenated at 45°C (50 lb/sq. inch) in the presence of palladium black (200 mg), which was freshly prepared before use. After 10 hours, the catalyst was changed to a new one (150 mg) and the reaction was continued for 10 hours. After removal of the catalyst, the solution was evaporated to give a pale yellow solid (266 mg). On paperchromatography with Solvent A, the solid showed three spots of Rf_{KM} 0.42 (main), 1 (minor, kanamycin) and 0.7 (minor). The aqueous solution of the solid was chromatographed on a column of Dowex 1×2 (OH form, 15×300 mm) with water. Kanamycin only was eluted by this procedure. The column was then washed with 0.1 N hydrochloric acid, the other two products (Rf 0.42 and 0.7) being eluted without separation. Evaporation of the eluate gave a solid (185 mg).

As all attempts to separate the two products by column chromatography was unsuccessful, the separation was performed by preparative paperchromatography. An aqueous solution of the solid (320 mg) was applied onto three sheets of paper (Toyo Roshi No. 525, 40×40 cm) and developed with Solvent A for 7 days. The zone containing the main

product (checked by ninhydrin coloration) was excised and the pieces were extracted with 0.1 N hydrochloric acid. The extracts were concentrated and the concentrate was charged on a column of Dowex 1×2 (OH form, 10×90 mm) and developed with water. The column was then washed with 0.1 N hydrochloric acid and the eluate containing the product was concentrated. Addition of 2 N hydrochloric acid to the concentrate to pH 1 followed by the addition of acetone gave an almost colorless solid (85 mg, 9 % overall yield from the starting substance); m. p. ~210°C (decomp.); $[\alpha]_D^{15}$ +95° (*c* 1, water). The substance had the same R*f*-value and antibacterial activity as that of **VI** obtained by the permanganate oxidation method. The minor substance (R*f* 0.73) was purified similarly; however, the substance showed no antibacterial activity for the microorganisms tested and the authors abandoned further investigation.

Hydrolysis of **VI**, **VII** and **VIII**: A small quantity of **VI**, **VII** or **VIII** in 6 N hydrochloric acid was heated, respectively, at 100°C for 2 hours. When the solutions were checked by paper chromatography with Solvent A and by ninhydrin coloration, each of the hydrolyzates showed two distinct spots corresponding to deoxystreptamine and 6-amino-6-deoxy-D-glucose (R*f* deoxystreptamine 1.5). Some other pale-colored spots appeared with tailing.

Acknowledgement

The authors wish to express their gratitude to Institute of Microbial Chemistry for bioassay, to Mr. S. NAKADA of our laboratory for microanalysis.

References

1) UMEZAWA, S.; T. TSUCHIYA & S. IRIYAMA: Synthesis of deoxykanamycin. J. Antibiotics, Ser. A 16 : 173~174, 1963; Studies of antibiotics and related substances. XXI. The synthesis of deoxy and chlorodeoxy derivatives of kanamycin. Bull. Chem. Soc. Japan. 38 : 1181~1186, 1965
2) UMEZAWA, S.; K. TATSUTA, T. TSUCHIYA & E. YAMAMOTO: Synthesis of kanamycin-6″-phosphate. J. Antibiotics, Ser. A 20 : 232~233, 1967; Studies of antibiotics and related substances. XXXI. The synthesis of kanamycin-6″-phosphate. Bull. Chem. Soc. Japan. 40 : 1972~1974, 1967
3) For a review, see K. HEYNS & H. PAULSEN: Selective catalytic oxidation of carbohydrates, employing platinum catalysts. Advances in Carbohydrate Chemistry. 17 : 169~221, 1962