BB-K 8, A NEW SEMISYNTHETIC AMINOGLYCOSIDE ANTIBIOTIC

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BB-K 8 is a new derivative of kanamycin acylated with L(−)-γ-amino-α-hydroxybutyric acid at the C-1 amino group of the 2-deoxystreptamine moiety. The details of the synthesis, involving a selective acylation of kanamycin, as well as the structural proof for BB-K 8 are described. BB-K 8 has antibacterial activity generally equal to kanamycin against kanamycin-sensitive organisms and is also active against kanamycin- and/or gentamicin-resistant organisms, including *Pseudomonas* strains. It gives good protection against experimental infections in mice with both kanamycin-sensitive and resistant organisms. BB-K 8 is not orally absorbed but gives high blood levels after parenteral administration and is excreted unchanged in the urine. BB-K 8 is less toxic than kanamycin in terms of acute intravenous LD_{50}.

The aminoglycoside antibiotics are valuable, widely-used chemotherapeutic agents which are limited in use primarily because of potential toxicity. Also, resistant strains have been reported in increasing numbers and simultaneously our knowledge of the mechanism of resistance has been expanding. We have been working with new natural and semisynthetic aminoglycosides to overcome these problems.

In our new antibiotic screening program we isolated from a bacterial culture an aminoglycoside antibiotic (Bu-1709) which was subsequently identified with butirosin[1~4]. A comparison of butirosin with ribostamycin*,[5,6] (Fig. 1) is interesting in that acylation of ribostamycin with L(−)-γ-amino-α-hydroxybutyric acid (L-HABA) at the C-1 amino group of deoxystreptamine moiety gives an improved antibiotic which inhibits some ribostamycin-kanamycin resistant organisms including *Pseudomonas* species (Table 1).

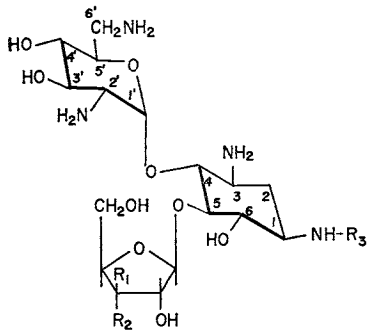
This observation and some of our theories as to the conformational structure of butirosin led us to investigate new types of modified aminoglycosides, one of which is BB-K 8, a new kanamycin derivative acylated with L-HABA at the C-1 amino group (Fig. 2).

Chemistry

There are four acylatable amino groups in the kanamycin molecule, two in the deoxystreptamine (DOS) part and one each in the 6-amino-6-deoxy-D-glucose (6-AG) and 3-amino-3-deoxy-D-glucose (3-AG) moieties. For convenience, these four amino groups are designated as N^1, N^2, N^3 and N^4 in the order of reactivity to acylating

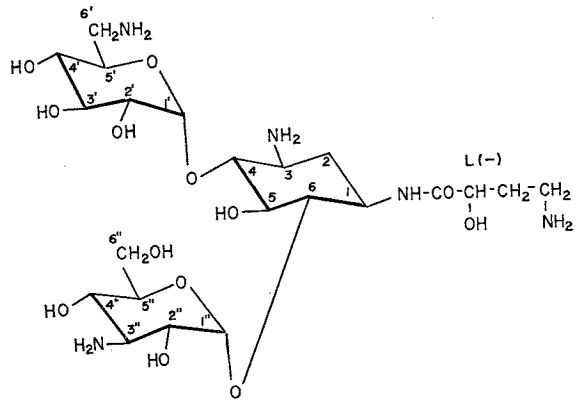
* Our ribostamycin sample (Bu-1709-DA_2) was obtained by alkaline hydrolysis of butirosin B.

Fig. 1. Structure of butirosins and ribostamycin.



	R_1	R_2	R_3
Butirosin A	OH	H	CO-CH(OH)-CH_2-CH_2-NH_2
Butirosin B	H	OH	CO-CH(OH)-CH_2-CH_2-NH_2
Ribostamycin	H	OH	H

Fig. 2. Structure of BB-K 8.



agent. The most reactive amino function, N^1, has been assumed to be the 6′-amino group in the 6-AG part, and this was confirmed by the fact that a 6′-acylamino kanamycin derivative* was obtained when kanamycin was treated with a mole of acylating agent. As anticipated, this derivative had little activity.

The selective acylation of kanamycin at the second most reactive amino function, N^2, was accomplished by reacting an N^1-blocked kanamycin with an acylating agent. The N^1-amino function was protected by the carbobenzoxy (Cbz) group which was subsequently removed by catalytic hydrogenolysis. Likewise, the N^3 and N^4 acylation products* were prepared from the N^1, N^2-di-blocked and N^1, N^2, N^3-tri-blocked kanamycin derivatives, respectively.

Table 1. Comparative antibacterial spectra of butirosin and ribostamycin

	MIC (mcg/ml)		
	Butirosin (Bu-1709)	Ribostamycin (Bu-1709 DA_2)	Kanamycin
S. aureus Smith	0.8	1.6	0.8
〃 A 20239	1.6	>100	100
E. coli Juhl	0.8	1.6	0.8
〃 K 12	0.4	0.8	0.8
〃 A 20665	0.4	>100	>100
〃 A 20635	50	>100	100
P. aeruginosa D 15	6.3	>100	25
〃 H 9	>100	>100	>100

Table 2. Physico-chemical properties of BB-K 8

Appearance	White crystalline powder
Melting point	203~204°C
Specific rotation	$[\alpha]_D^{23}$ +99° (*c* 1.0, H_2O)
Anal. Calcd. for $C_{22}H_{43}N_5O_{13}\cdot{}^3/_2H_2O$:	C 43.13, H 7.57, N 11.43
Found:	C 42.88, H 7.93, N 11.04
Infrared spectrum (KBr)	Major absorption bands at 3350, 2930, 1640, 1580, 1350 cm^{-1}
NMR spectrum (100 MHz in D_2O, δ in ppm, J in Hz)	1.1~2.3 (4 H, m), 2.6~3.95 (19 H, m), 4.13 (1 H, d-d, J=4.5 & 8.5), 4.97 (1 H, d, J=3.5), 5.21 (1 H, d, J=3.5).

The N^2-acylated products yielded an active series of considerable interest. BB-K 8 is the N^2-acylation product of kanamycin with L(−)-γ-amino-α-hydroxybutyric acid (L-HABA).

Synthesis of BB-K 8 (Scheme 1)

The N^1-carbobenzoxylation of kanamycin (**1**) at the 6′-amino group was achieved

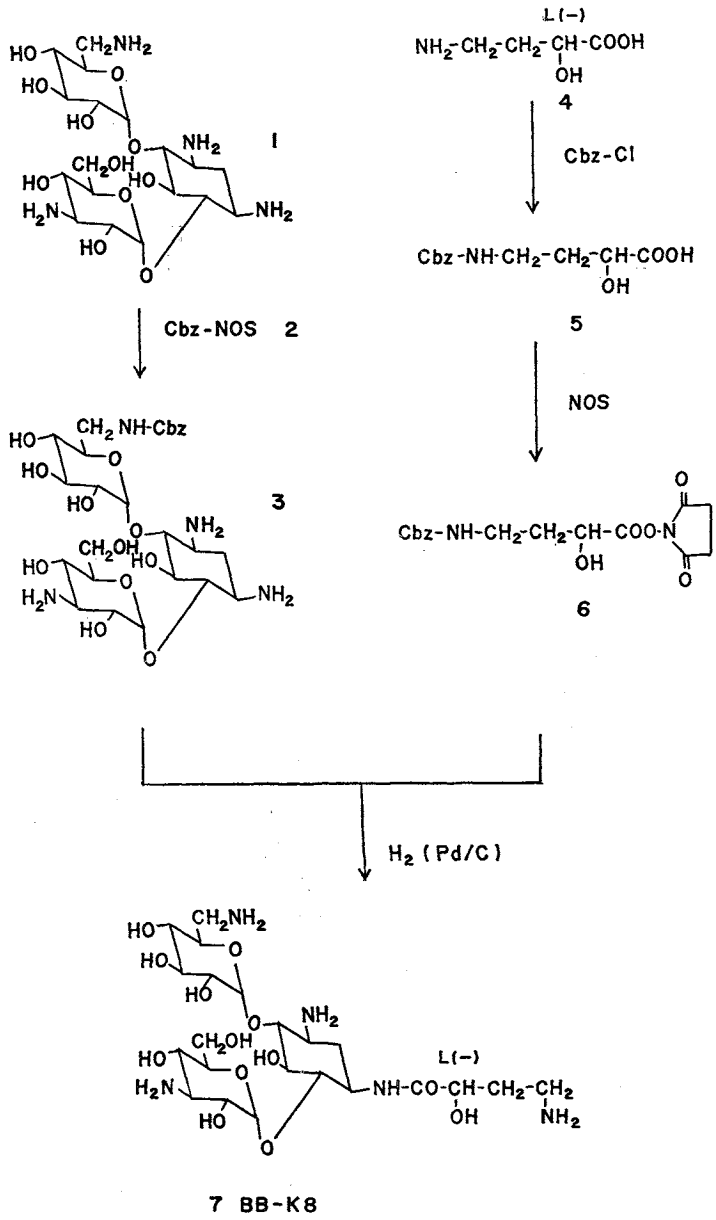
* These isomers of BB-K 8 will be reported later in a separate paper.

by the activated ester method using N-(benzyloxycarbonyloxy) succinimide (2, Cbz-NOS). The reaction product was purified by ion-exchange chromatography on Amberlite CG-50 to give 6′-N-benzyloxycarbonyl kanamycin (3, 6′-Cbz-kanamycin).

The acylating agent was prepared by protecting the γ-amino group of L-HABA (4) with carbobenzoxy chloride, followed by reaction with N-hydroxy-succinimide to yield the active ester of N-Cbz-protected-L-HABA (6).

The N^2-acylation of N^1-Cbz-kanamycin (3) was carried out by an equimolar reaction with compound 6 at room temperature. The reaction mixture was subjected to hydrogenolysis to remove protecting groups at both the N^1 and the γ-amino in the acyl side chain. The crude BB-K 8 thus obtained was purified by column chromatography on Amberlite CG-50 with aqueous ammonia. The active fractions were combined, evaporated *in vacuo* and the residue was crystallized from methanol-isopropanol to yield needle crystals of BB-K 8 base. The physico-chemical properties of BB-K 8 are shown in Table 2.

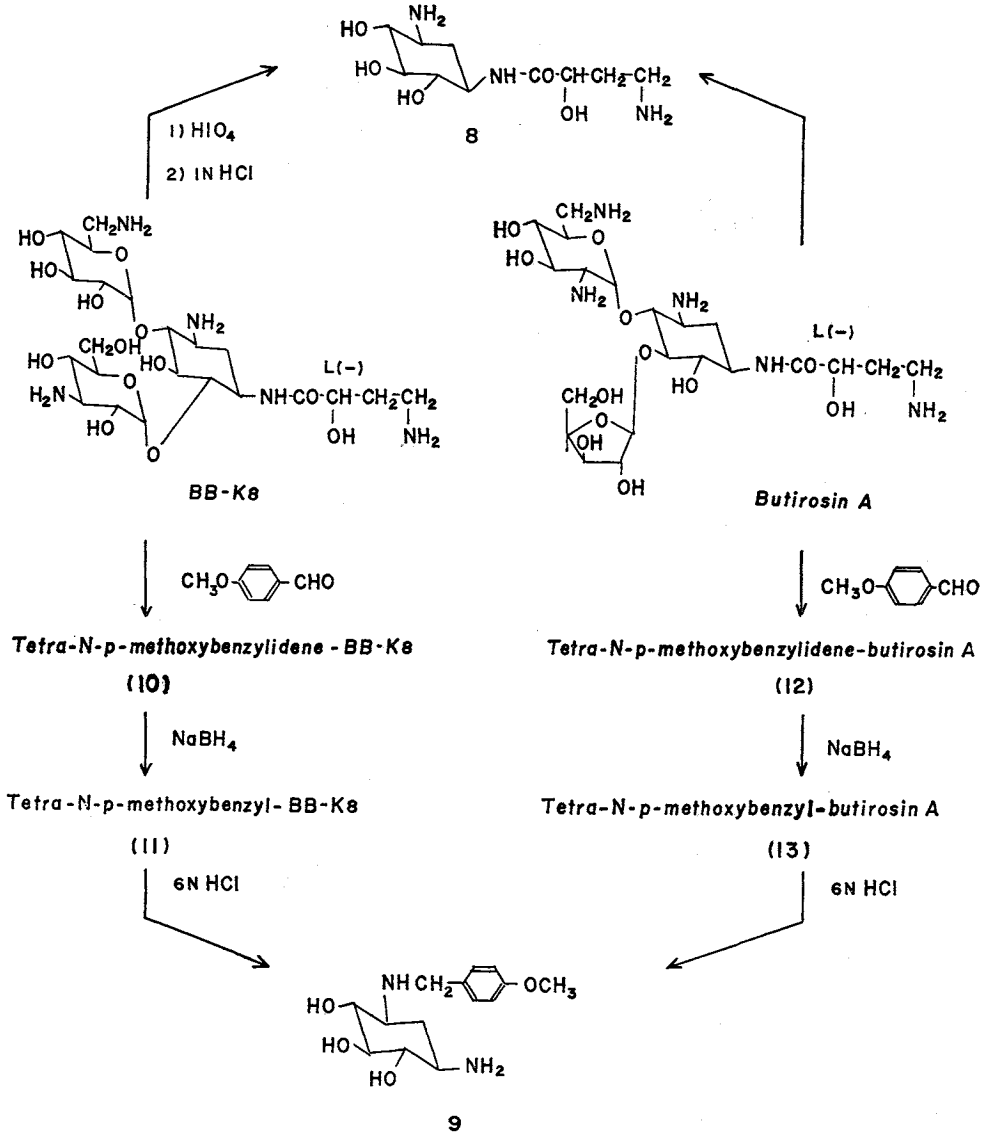
Scheme 1. Preparation of BB-K 8.



Structure of BB-K 8 (Scheme 2)

The site of acylation with L-HABA in the BB-K 8 molecule must be in either the 2-deoxystreptamine or the 3-amino-3-deoxy-D-glucose (3-AG) moiety. Periodate oxidation studies[7] on BB-K 8 showed considerably slower consumption of the periodate reagent than seen with kanamycin. However, BB-K 8 consumed 4 moles of periodate after about 24 hours, and accordingly the side chain acid must be attached

Scheme 2. Degradation of BB-K 8 and butirosin A.



to either one of the two amino groups in the deoxystreptamine moiety.

The position of the amide linkage in the deoxystreptamine moiety has been determined by comparative degradation studies of BB-K 8 with butirosin as shown in Scheme 2. BB-K 8 was treated with periodic acid to oxidize the 6-AG and 3-AG moieties and then hydrolyzed with 1 N HCl to afford the L-HABA-acylated deoxystreptamine (8). Butirosin yielded the same degradation product[4] as shown by TLC and ORD, proving 8 to be the 1-N-acyl deoxystreptamine.

Further unequivocal evidence for the site of acylation with L-HABA in BB-K 8 was furnished by the following sequence of reactions. The SCHIFF's base of BB-K 8 with *p*-methoxybenzaldehyde was reduced with sodium borohydride and then vigorously hydrolyzed with 6 N HCl to give 3-N-*p*-methoxybenzyl-2-deoxystreptamine (**9**), which was identical by ORD and circular dichroism (CD) with the corresponding

product obtained from butirosin A. Both compounds showed a double positive COTTON effect curve with two peaks at 280 and 332 nm in ORD, and with two positive peaks at 273 and 223 nm in CD. The L-HABA in butirosin is at the C-1 amino group of the deoxystreptamine moiety and the same position is established for BB-K 8, since attachment of the acyl residue at the C-3 amino group would yield the enantiomer of the butirosin product, which should have the opposite signs in the ORD and CD curves to those of compound **9**. Thus, the structure of BB-K 8 is 1-N-[L(−)-γ-amino-α-hydroxybutyryl] kanamycin A.

Experimental

N-(Benzyloxycarbonyloxy) succinimide (**2**)

N-Hydroxysuccinimide (23 g, 0.2 mole) was dissolved in a solution of 9 g (0.22 mole) of sodium hydroxide in 200 ml of water. To the stirred solution was added dropwise 34 g (0.2 mole) of carbobenzoxy chloride with water-cooling and then the mixture was stirred at room temperature for 4 hours to separate the carbobenzoxy derivative which was collected by filtration, washed with water and air-dried. Yield 41.1 g (82 %). Recrystallization from benzene-*n*-hexane (10 : 1) gave colorless prisms melting at 78~79°C.

6′-N-Benzyloxycarbonyl kanamycin A (**3**)

A solution of 42.5 g (90 mmoles) of kanamycin A free base (**1**) in 450 ml of water and 500 ml of dimethylformamide (DMF) was cooled below 0°C and stirred vigorously. To the solution was added dropwise over a period of about two hours a solution of 22.4 g (90 mmoles) of **2** in 500 ml of DMF. The mixture was stirred at −10°~0°C overnight and then at room temperature for one day. The reaction mixture was evaporated under reduced pressure below 50°C. The oily residue was dissolved in a mixture of 500 ml water and 500 ml butanol, the mixture being filtered to remove insoluble material, and separated into two layers. The butanol and aqueous layers were treated with butanol-saturated water (500 ml × 2) and water-saturated butanol (500 ml × 2), respectively, using a technique similar to counter-current distribution. The three aqueous layers were combined and evaporated to dryness under reduced pressure to give an oily residue, a part of which crystallized on standing at room temperature. To the residue including the crystals was added about 100 ml of methanol, which dissolved the oil and separated it from the crystals. After adding about 300 ml of ethanol, the mixture was kept at room temperature overnight to give a crystalline mass which was collected by filtration. It weighed 44 g. The product contained a small amount of kanamycin A as indicated by thin-layer chromatography using *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) as the solvent system and ninhydrin as the spray reagent.

The crude product was dissolved in 300 ml of water and chromatographed on a column (30 mm diameter) of CG-50 ion-exchange resin (NH_4^+ form, 500 ml). The column was irrigated with 0.1 N ammonium hydroxide solution and the eluate was collected in 10-ml fractions. The desired product was contained in tube numbers 10~100, which were combined and evaporated to dryness under reduced pressure to give 24.6 g (45 %) of white powder **3**, mp 204~212°C (dec.), $[\alpha]_D^{25}$ +116° (*c* 2, H_2O). TLC (S-114*, ninhydrin): Rf 0.23.

Anal. Calcd. for $C_{26}H_{42}N_4O_{13}\cdot H_2O$: C 49.05, H 6.97, N 8.80
Found : C 48.98, H 7.20, N 8.90

L(−)-γ-Benzyloxycarbonylamino-α-hydroxybutyric acid (**5**)

L(−)-γ-Amino-α-hydroxybutyric acid (**4**) (7.4 g, 0.062 mole) was added to a solution of 5.2 g (0.13 mole) of sodium hydroxide in 50 ml of water. To the stirred solution was added dropwise at 0~5°C over a pariod of 0.5 hour, 11.7 g (0.068 mole) of carbobenzoxy chloride and the mixture was stirred for one hour at the same temperature. The reaction mixture

* S-114 : MeOAc - *n*-PrOH - conc. NH_4OH (45 : 105 : 60)

was washed with 50 ml of ether, adjusted to pH 2 with dilute hydrochloric acid and extracted with four 80-ml portions of ether. The ethereal extracts were combined, washed with a small amount of saturated sodium chloride solution, dried with anhydrous sodium sulfate and filtered. The filtrate was evaporated *in vacuo* and the resulting residue was crystallized from benzene to give 11.6 g (74 %) of colorless plates **5**, mp 78.5~79.5°C, $[\alpha]_D^{22}$ −4.5° (*c* 2.0, MeOH). IR (KBr): $\nu_{C=O}$ 1740, 1690 cm^{-1}. NMR (acetone-d_6): δ (in ppm) 2.0 (2 H, m), 3.29 (2 H, d-d, J=6.7 and 12 Hz), 4.16 (1 H, d-d, J=4.5 and 8 Hz), 4.99 (2 H, s), 6.2 (2 H, broad), 7.21 (5 H, s).

Anal. Calcd. for $C_{12}H_{15}NO_5$: C 56.91, H 5.97, N 5.53
Found: C 56.66, H 5.97, N 5.47

N-Hydroxysuccinimide ester of L(−)-γ-benzyloxycarbonylamino-α-hydroxybutyric acid(**6**)

A solution of 10.6 g (0.042 mole) of **5** and 4.8 g (0.042 mole) of N-hydroxysuccinimide in 200 ml of ethyl acetate was cooled to 0°C and then 8.6 g (0.042 mole) of dicyclohexylcarbodiimide was added. The mixture was kept overnight in a refrigerator. The dicyclohexylurea which separated was filtered off and the filtrate was concentrated to about 50 ml under reduced pressure to give colorless crystals of **6** which were collected by filtration; 6.4 g, mp 121~122.5°C. The filtrate was evaporated to dryness *in vacuo* and the crystalline residue was washed with 20 ml of a benzene – *n*-hexane mixture to give an additional amount of **6**. The total yield was 13.4 g (92 %). $[\alpha]_D^{23}$ +1.5° (*c* 2, $CHCl_3$). IR (KBr): $\nu_{C=O}$ 1810, 1755, 1740, 1680 cm^{-1}. NMR (acetone-d_6): δ (in ppm) 2.0 (2 H, m), 2.83 (4 H, s), 3.37 (2 H, d-d, J=6.5 and 12.5 Hz), 4.56 (1 H, m), 4.99 (2 H, s), 6.3 (2 H, broad), 7.23 (5 H, s).

Anal. Calcd. for $C_{16}H_{18}N_2O_7$: C 54.85, H 5.18, N 8.00
Found: C 54.75, H 5.21, N 8.13

BB-K 8, 1-N-[L(−)-γ-Amino-α-hydroxybutyryl] kanamycin A (**7**)

A solution of 1.6 g (4.6 mmoles) of **6** in 40 ml of ethyleneglycol dimethyl ether (DME) was added dropwise to a stirred solution of 2.6 g (4.2 mmoles) of **3** in 40 ml of 50 % aqueous DME and the mixture was stirred overnight at room temperature. The reaction mixture was evaporated under reduced pressure. The residue was dissolved in 40 ml of 50 % aqueous dioxane and a small amount of insoluble material was removed by filtration. To the filtrate was added 0.8 ml of glacial acetic acid and 1 g of 10 % palladium-on-charcoal and the mixture was hydrogenated at room temperature for 24 hours in a Parr hydrogenation apparatus. The reaction mixture was filtered to remove the palladium catalyst and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 30 ml of water and chromatographed on a column of CG-50 ion-exchange resin (NH_4^+ type, 50 cm × 1.8 cm). The column was washed with 200 ml of water and then eluted with 800 ml of 0.1 N NH_4OH, 500 ml of 0.2 N NH_4OH and finally 500 ml of 0.5 N NH_4OH. The eluate was collected in 10-ml fractions and monitored by ninhydrin spot test, disc assay (*Bacillus subtilis* and *Pseudomonas aeruginosa*) and TLC on silica gel (S-110*, ninhydrin). Fractions 146~154 showed a ninhydrin-positive spot at Rf 0.16 and were active against *P. aeruginosa* strain A 9843. They were combined, evaporated *in vacuo* and finally freeze-dried to give 552 mg of crude BB-K 8 (**7**) (22 % based on **3**).

A solution of 450 mg of the crude **7** in 5 ml was neutralized in 1 N HCl and adsorbed on a column of CG-50 (NH_4^+, 6 ml), which was washed with 20 ml of water and eluted with 0.5 N NH_4OH. The eluate was collected in 5-ml fractions. Tubes Nos. 1~7 were ninhydrin-positive. The heart cut, tube Nos. 2~4, was evaporated *in vacuo* to give an oily syrup, which was dissolved in 2 ml of methanol. About 1 ml of isopropanol was added to the solution until incipient turbidity and, upon standing for 3 hours at room temperature, colorless crystals separated. After standing in a refrigerator for 3 hours the crystals were filtered, washed with ethanol and dried overnight *in vacuo* over phosphorus pentoxide to afford 350 mg of BB-K 8 free base, mp 203~204°C, $[\alpha]_D^{25}$ +99° (*c* 1.0, H_2O).

* S-110 : $CHCl_3$ - MeOH - 28 % NH_4OH - H_2O (25 : 25 : 3 : 47)

IR (KBr): 3350, 2930, 1640, 1580, 1350 cm^{-1}, NMR (100 MHz in D_2O): δ (in ppm), 1.1~2.3 (4 H, m), 2.6~3.95 (19 H, m), 4.13 (1 H, d-d, J=4.5 & 8.5 Hz), 4.97 (1 H, d, J=3.5 Hz, anomeric H), 5.21 (1 H, d, J=3.5 Hz, anomeric H).

Compound	Periodate consumed (mole)				
	1 hr	3 hr	5 hr	7 hr	24 hr
BB-K 8	2.3 2.0	2.9 2.5	3.2 2.8	3.5 3.1	4.2 3.7
Kanamycin A	2.9 2.7	3.4 3.2	3.7 3.5	3.9 3.7	4.4 4.2

Anal. Calcd. for $C_{22}H_{43}N_5O_{13}\cdot{}^3/_2\,H_2O$: C 43.13, H 7.57, N 11.43
Found: C 42.88, H 7.94, N 11.04

Periodate oxidation of BB-K 8

Periodate oxidation of BB-K 8 along with kanamycin A was carried out according to the usual procedure[7]. The results of two runs are given in the above table.

1-N-L(−)-γ-Amino-α-hydroxybutyryl-2-deoxystreptamine (8) from BB-K 8

To a stirred solution of 3.0 g (5.1 mmoles) of BB-K 8 in 880 ml of water was added a solution of 9.3 g (40.9 mmoles) of periodic acid in 100 ml of water. The mixture was kept for 15 hours at room temperature, neutralized with 8.2 g (26 mmoles) of barium hydroxide, treated with 2 g of barium carbonate and filtered to remove the precipitates. The filtrate was concentrated to 50 ml and the concentrate was heated with 50 ml of 2 N HCl. After cooling, the mixture was neutralized with conc. NH_4OH and charged on a column of CG-50 (NH_4^+, 80 ml), which was washed with 300 ml of water and eluted successively with 630 ml of 0.05 N NH_4OH, 540 ml of 0.1 N NH_4OH, 530 ml of 0.2 N NH_4OH and 1620 ml of 0.5 N NH_4OH. The eluate was collected in 10-ml fractions. Fractions 221~310, which had Rf 0.18 and 0.27 by TLC on a silica gel plate (S-110, ninhydrin) were combined, evaporated *in vacuo* and lyophilized to give crude **8**, which was rechromatographed on CG-50 (NH_4^+) to give 325 mg (24 %) of **8**, mp 158~160°C (dec.). TLC (S-110, ninhydrin): Rf 0.27. IR (KBr): 3400 (broad), 1640, 1550, 1090, 1020 cm^{-1}. NMR (D_2O): δ (in ppm from HOD), 0.43 (1 H, d-d, J=6.8 & 4.5 Hz, $-COCH\langle^{O-}$), 1.56 (2 H, t, J=7.5 Hz, $-CH_2N\langle$), 2.4~2.9 (3 H, m, 2-H_{eq} & C-CH_2-C), 3.26 (1 H, q, J=12 Hz, 2-H_{ax}). ORD (*c* 0.1, H_2O): negative COTTON effect curve, $[\alpha]_{220}^{trough}-960°$, $[\alpha]_D-20°$.

Anal. Calcd. for $C_{10}H_{21}N_3O_5\cdot{}^1/_2\,H_2CO_3$: C 42.85, H 7.53, N 14.28
Found: C 43.17, H 7.50, N 13.97

N-Acetylation of 115 mg of **8** with acetic anhydride in methanol gave 117 mg (78 %) of N,N′-diacetyl derivative of **8**, which was crystallized from ethanol, mp 221~223°C (lit.[4] 211~213°C). IR (KBr): $\nu_{C=O}$ 1640 cm^{-1}.

Anal. Calcd. for $C_{14}H_{25}N_3O_7$: C 48.41, H 7.25, N 12.10
Found: C 48.23, H 7.09, N 11.75

1-N-L(−)-γ-Amino-α-hydroxybutyryl-2-deoxystreptamine (8) from butirosin A

Periodic acid oxidation of 3.0 g (5.4 mmoles) of butirosin A, followed by hydrolysis with 1 N HCl in the same way as described above gave 338 mg (24 %) of **8**, mp 158~160°C (dec.), which was identical with **8** from BB-K 8 in TLC, IR, NMR and ORD.

Anal. Calcd. for $C_{10}H_{21}N_3O_5\cdot{}^1/_2\,H_2CO_3$: C 42.85, H 7.53, N 14.28
Found: C 43.26, H 7.38, N 14.07

Acetylation with acetic anhydride in methanol gave N,N′-diacetate (mp 221~223°C, lit.[4]. mp 211~213°C) which showed no depression in a mixture melting point with the corresponding acetate from BB-K 8.

Tetra-N-*p*-methoxybenzylidene BB-K 8 (**10**)

To a stirred solution of 1.17 g (2.0 mmoles) of BB-K 8 and 160 mg (4.0 mmoles) of sodium hydroxide in 10 ml of water was added a solution of 1.63 g (12 mmoles) of *p*-methoxybenzaldehyde in 10 ml of ethanol. The mixture was stirred for 30 minutes at room temperature and evaporated *in vacuo* to deposit **10** which was washed throughly with water and dried *in vacuo* over phosphorus pentoxide to yield 1.77 g (84 %) of **10**, mp 157~160°C.

Anal. Calcd. for $C_{54}H_{67}N_5O_{17}$: C 61.29, H 6 38, N 6.62
Found: C 60.88, H 6.51, N 6.50

Tetra-N-*p*-methoxybenzyl BB-K 8 (**11**)

To a stirred solution of 1.7 g (1.5 mmoles) of **10** in 50 ml of THF - MeOH mixture (3 : 2) was added 235 mg (6.2 mmoles) of sodium borohydride. The mixture was stirred for 2 hours at room temperature and filtered to remove a small amount of insoluble material. The filtrate was concentrated to about 3 ml and mixed with 40 ml of water. The precipitate formed was isolated by decantation and reprecipitated from MeOH - water (3 : 40) to give 1.43 g (89 %) of **11**, mp 133~138°C.

Anal. Calcd. for $C_{54}H_{75}N_5O_{17}$: C 60.83, H 7.09, N 6.57
Found: C 60.06, H 7.10, N 6.70

3-N-*p*-Methoxybenzyl-2-deoxystreptamine (**9**) from **11**

A suspension of 4.95 g (4.63 mmoles) of **11** in 150 ml of 6 N hydrochloric acid was refluxed for 6 hours. The mixture was evaporated *in vacuo* to give an oily residue, which was dissolved in 50 ml of water. The aqueous solution was adjusted to pH 10 with conc. ammonia and filtered to remove insoluble material. The filtrate was neutralized with 6 N hydrochloric acid and charged on a column of CG-50 (NH_4^+, 60 ml), which was washed with 170 ml of water and eluted with 0.02 N ammonia. The eluate was collected in 10-ml fractions. Tube Nos. 73~130 which had ninhydrin-positive spots at Rf 0.34, 0.45 (main), 0.55, 0.67 by TLC (silica gel plate, S-114 system), were combined, evaporated *in vacuo* and freeze-dried to give 476 mg of crude **9**. The crude **9** (336 mg) was rechromatographed on a column of IR-120 (NH_4^+, 45 ml) which was eluted with 180 ml of 0.05 N, 550 ml of 0.1 N, 660 ml of 0.2 N and 550 ml of 0.5 N ammonia. The eluate was collected in 10-ml fractions. Tube Nos. 81~195 which showed a single spot at Rf 0.45 by TLC were combined, evaporated *in vacuo* and freeze-dried to give 261 mg of **9**, mp 74~76°C. IR (KBr): 3350 (broad), 1610, 1510, 1250, 1030, 815 cm^{-1}. NMR (D_2O): ppm from HOD, 3.50 (1 H, q, J=12 Hz, 2-H_{ax}), 2.48 (1 H, d-t, J=12.0 and 3.8 Hz, 2-H_{eq}), 1.7~2.4 (2 H, m, >CH-N), 1.1~1.6 (3 H, m, >CH-OH), 1.01 (2 H, s, $-CH_2-$Ar), 0.9 (3 H, s, OCH_3), −2.18 and −2.53 (4 H, AB, q, J=8.7 Hz, ring H). ORD (*c* 0.1, H_2O): Positive COTTON effect curve, $[\alpha]_{232}^{peak}$+1100°, $[\alpha]_{270}^{trough}$+200°, $[\alpha]_{280}^{peak}$+320°. CD: $[\theta]_{223}$+4790, $[\theta]_{273}$+259.

Anal. Calcd. for $C_{14}H_{22}N_2O_4 \cdot {}^1/_2\ H_2O$: C 57.72, H 7.96, N 9.62
Found: C 57.43, H 8.00, N 9.67

Tetra-N-*p*-methoxybenzylidene butirosin A (**13**)

Butirosin A (5 g, 9.0 mmoles) was reacted with *p*-methoxybenzaldehyde in the same way as BB-K 8 to afford 6.25 g (67 %) of **13**, mp 185~188°C.

Anal. Calcd. for $C_{53}H_{65}N_5O_{16} \cdot 2\,H_2O$: C 59.82, H 6.54, N 6.58
Found: C 60.06, H 7.12, N 6.51

Tetra-N-*p*-methoxybenzylbutirosin A (**14**)

The SCHIFF base (**13**) (5.17 g, 5.0 mmoles) was reduced with sodium borohydride in the same way as **10** to give 5.0 g (97 %) of **14**, mp 129~132°C.

Anal. Calcd. for $C_{53}H_{73}N_5O_{16} \cdot H_2O$: C 60.38, H 7.17, N 6.64
Found: C 60.03, H 7.25, N 6.35

3-N-*p*-Methoxybenzyl-2-deoxystreptamine (**9**) from **14**

Compound **14** was hydrolyzed in the same way as **11** followed by CG-50 (NH_4^+) and IR-120 (NH_4^+) column chromatography to yield 476 mg (36 %) of **9**, mp 73~76°C. IR (KBr): 3350 (broad), 1610, 1510, 1250, 1030, 815 cm^{-1}, NMR (D_2O): ppm from HOD, 3.51 (1 H, q, J=12 Hz, 2-H_{ax}), 2.46 (1 H, d-t, J=12 and 3.8 Hz, 2-H_{eq}), 1.75~2.25 (2 H, m, CH-N), 1.01 (2 H, s, CH_2-Ar), 0.90 (3 H, s, OCH_3), −2.17 and −2.54 (4 H, ABq, J=8.7 Hz, ring H). ORD (*c* 0.1, H_2O): positive COTTON effect curve, $[\alpha]_{232}^{peak}$+1800°, $[\alpha]_{270}^{trough}$+400°, $[\alpha]_{280}^{peak}$+520. CD: $[\theta]_{223}$+4820, $[\theta]_{273}$+402. The admixture of **9** from BB-K8 and **9** from butirosin A showed no depression in a mixed melting point determination.

Table 3. Antibacterial spectra of BB-K 8 and kanamycin

Test organism	BBRI Code	MIC (mcg/ml)		Ratio KM/BB-K 8
		BB-K 8	Kanamycin	
Escherichia coli NIHJ	Ec-1	0.4	0.8	2
〃 〃 Juhl	Ec-3	0.8	1.6	2
〃 〃 A 15169	Ec-4	0.4	0.8	2
〃 〃 A 20363 (ML-1630)	Ec-5	0.8	>100	>128
〃 〃 A 9844	Ec-6	0.4	0.8	2
〃 〃 A 20365	Ec-7	0.1	50	512
〃 〃 K 12	Ec-8	0.2	0.8	4
〃 〃 A 20664	Ec-9	0.4	6.3	16
〃 〃 A 20665	Ec-10	0.2	100	512
〃 〃 A 9535	Ec-44	0.8	1.6	2
〃 〃 A 15148	Ec-45	0.8	1.6	2
〃 〃 A 15164	Ec-46	0.8	1.6	2
〃 〃 A 15170	Ec-47	0.8	1.6	2
〃 〃 A 20102	Ec-48	0.8	1.6	2
〃 〃 W 677	Ec-52	0.4	0.8	2
〃 〃 JR 66/W 677	Ec-53	0.8	>100	>128
Klebsiella pneumoniae D 11	Kp-1	0.1	0.2	2
〃 〃 Type 22, # 3038	Kp-8	0.8	>100	>128
Klebsiella sp. A 9661	Kx-1	1.6	1.6	1
〃 A 9662	Kx-2	1.6	1.6	1
Proteus vulgaris A 9436	Pv-1	0.2	0.4	2
〃 〃 A 9526	Pv-2	0.4	0.4	1
〃 〃 A 9699	Pv-3	1.6	0.8	1/2
〃 〃 ATCC 9920	Pv-4	0.8	0.4	1/2
〃 〃 A 9539	Pv-5	0.4	0.4	1
〃 〃 A 9716	Pv-6	1.6	1.6	1
Proteus morganii A 9553	Pg-1	0.8	0.8	1
〃 〃 A 20031	Pg-2	0.8	0.8	1
〃 〃 A 9636	Pg-3	3.1	0.8	1/4
〃 〃 A 15153	Pg-4	3.1	0.8	1/4
〃 〃 A 15166	Pg-5	0.8	0.8	1
〃 〃 A 20455	Pg-6	1.6	1.6	1
〃 〃 A 20457	Pg-7	1.6	1.6	1
Proteus inconstans A 20615	Pn-1	1.6	1.6	1
Proteus rettgeri A 9637	Pr-2	0.8	0.4	1/2
Proteus mirabilis A 9554	Pm-1	0.8	0.8	1
〃 〃 A 9900	Pm-2	0.8	0.8	1
〃 〃 A 20119	Pm-3	1.6	1.6	1
〃 〃 A 20454	Pm-4	1.6	1.6	1
Serratia marcescens A 20019	Sm-1	0.8	1.6	2
〃 〃 A 20335	Sm-2	0.8	100	128
Pseudomonas aeruginosa D 15	Pa-1	1.6	25	16
〃 〃 A 9923	Pa-2	6.3	100	16
〃 〃 A 9930	Pa-3	0.4	12.5	32
〃 〃 H 9	Pa-4	6.3	>100	> 16
〃 〃 A 15150	Pa-5	6.3	100	16
〃 〃 A 15194	Pa-6	3.1	50	16
〃 〃 A 20479	Pa-10	6.3	>100	> 16
〃 〃 A 20616	Pa-11	12.5	100	8
〃 〃 A 20653	Pa-12	12.5	>100	> 8
〃 〃 A 9843	Pa-13	6.3	50	8
〃 〃 A 20717	Pa-15	6.3	50	8
〃 〃 A 20718 (# 130)	Pa-16	6.3	50	8

(*to be continued*)

(*continued*)

Test organism	BBRI Code	MIC (mcg/ml)		Ratio KM/BB-K 8
		BB-K 8	Kanamycin	
Pseudomonas sp. A 20355	Px-1	0.8	12.5	16
〃 〃 A 20358	Px-2	6.3	25	4
〃 〃 A 20368	Px-3	12.5	50	4
〃 〃 A 20598	Px-4	12.5	25	2
〃 〃 A 20600	Px-5	6.3	25	4
〃 〃 A 20603	Px-6	12.5	>100	8
〃 〃 A 20618	Px-7	100	100	1
〃 〃 A 20594	Px-8	12.5	100	8
Staphylococcus aureus FDA 209 P	Sa-1	0.8	0.8	1
〃 〃 Smith	Sa-2	0.4	0.4	1
〃 〃 #193	Sa-3	0.8	0.4	1/2
〃 〃 209 P	Sa-4	1.6	1.6	1
〃 〃 209 P	Sa-5	0.2	0.2	1
〃 〃 209 P	Sa-6	0.2	0.2	1
〃 〃 209 P	Sa-7	0.2	0.8	4
〃 〃 #52-34	Sa-8	0.4	0.8	2
〃 〃 #193	Sa-9	3.1	1.6	1/2
〃 〃 A 20239	Sa-10	1.6	100	64
Sarcina lutea PCI-1001	Sl-1	1.6	6.3	4
Micrococcus flavus	Mf-1	0.2	0.8	4
Bacillus mycoides strain 0	Bm-1	0.2	0.8	4
Bacillus sphaericus #122	Bp-1	0.2	0.8	4
Bacillus cereus ATCC 10702 A	Bc-1	0.4	0.8	2
Bacillus subtilis A 9506	Bs-2	0.05	0.05	1
Streptococcus pyogenes S-23	Sp-1	12.5	12.5	1
〃 〃 Dick	Sp-2	3.1	3.1	1
〃 〃 A 9604	Sp-3	12.5	12.5	1
〃 〃 A 20065	Sp-4	12.5	12.5	1
Diplococcus pneumoniae Type 2	Dp-1	12.5	12.5	1
Mycobacterium 607	M6-1	0.4	0.8	2
〃 607*	M6-2	>100	>100	—
〃 607*,**	M6-3	>100	>100	—
Mycobacterium phlei	Mp-1	0.2	0.4	2
Mycobacterium ranae	Mr-1	0.2	0.4	2
Mycobacterium tuberculosis $H_{37}Rv$	Mt-1	0.6	1.6	2.7
〃 〃 Schacht**	Mt-2	0.2	0.6	3
〃 〃 Sekiya*	Mt-3	>100	>100	—
〃 〃 Ohyabu*	Mt-4	>100	>100	—
〃 〃 Ogawa*	Mt-5	10.5	50	4.8
〃 〃 Arikawa	Mt-6	1.3	1.0	0.8
〃 〃 Fujiwara	Mt-7	0.3	0.7	2.3
〃 〃 Kamio	Mt-8	0.8	1.4	1.8
〃 〃 Nakamura	Mt-9	0.4	1.1	2.7
〃 〃 Ohno	Mt-10	1.3	5.3	4.1
〃 〃 Tarumi	Mt-11	1.1	5.3	4.8

* kanamycin-resistant ** streptomycin-resistant

Biological Activity

Antibacterial Activity *In Vitro*

The minimal inhibitory concentration (MIC) of BB-K 8 was determined by a twofold dilution method for 41 strains of *Enterobacteriaceae* (*E. coli*, *Klebsiella*, *Proteus* and *Serratia*), 20 strains of *Pseudomonas* species, 21 strains of gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Diplococcus pneumoniae* and others) and

16 strains of *Mycobacterium* including 5 laboratory strains and 11 clinical isolates of *M. tuberculosis*. The MIC against tubercle bacilli was determined by a serial tube dilution method using DUBOS liquid medium and other *Mycobacterium* strains were tested on plates containing No. 1001 agar medium*. Strains of *S. pyogenes* and *D. pneumoniae* were tested on 10 % horse blood agar and all other organisms on Nutrient Agar (Eiken) plates. Generally 10^{-4} dilutions of overnight cultures were used as the inocula except for strains of *Mycobacterium*, *S. pyogenes* and *D. pneumoniae* for which 10^{-3} dilutions were used.

The MICs of BB-K 8 and kanamycin are shown in Table 3. The last column of Table 3 shows the ratio of the kanamycin MIC to that of BB-K 8, and the data are further compiled in Tables 4 and 5 to show the distribution of MIC ratios of the test organisms. It can be seen from these results that BB-K 8 is equally active or even more active (especially against *E. coli* strains) than kanamycin against the kanamycin-sensitive organisms except for 5 of 19 *Proteus* species to which kanamycin showed somewhat

Table 4. Relative *in vitro* activity of BB-K 8 and kanamycin against Gram-negative bacteria

	No. of strains	MIC ratio (kanamycin/BB-K 8)						
		1/4	1/2	1	2	4	8	16
E. coli	16	0	0	0	10	1	0	5
Klebsiella	4	0	0	2	1	0	0	1
Proteus	19	2	3	13	1	0	0	0
Serratia	2	0	0	0	1	0	0	1
Pseudomonas	20	0	0	1	1	3	6	9
Total	61	2	3	16	14	4	6	16

Table 5. Relative *in vitro* activity of BB-K 8 and kanamycin against Gram-positive bacteria

	No. of strains	MIC ratio (kanamycin/BB-K 8)						
		1/4	1/2	1	2	4	8	16
S. aureus	10	0	2	5	1	1	0	1
S. pyogenes	4	0	0	4	0	0	0	0
D. pneumoniae	1	0	0	1	0	0	0	0
Miscellaneous	6	0	0	1	1	4	0	0
Total	21	0	2	11	2	5	0	1

Table 6. Activity of BB-K 8 against resistant organisms producing aminoglycoside-inactivating enzymes

Organism	Code	Original strains	MIC (mcg/ml)					Inactivating enzymes		Reference
			BB-K 8	KM	SM	NM	GM	Substrate*	Mechanism	
E. coli	Ec-5	A 20363 (ML-1630)	0.8	>100	25	100	0.8	KM	3′-phosphorylation	10)
E. coli	Ec-9	NR79/W677	0.4	6.3	6.3	3.1	0.8	KM	6′-acetylation	11), 12)
E. coli	Ec-10	JR35/C600	0.2	100	25	50	0.4	KM,NM	phosphorylation	13)
								SM	3″-phosphorylation	13)
E. coli	Ec-53	JR66/W677	0.8	>100	100	100	25	KM,NM,SM	phosphorylation	14)
								GM	adenylation	14)
								DKB	2″-adenylation	15)
K. pneumoniae	Kp-8	Type 22, #3038	0.8	>100	>100	>100	50	KM	phosphorylation	14), 16)
								GM	adenylation	14)
P. aeruginosa	Pa-4	H 9	6.3	>100	>100	50	12.5	KM,NM	3′-phosphorylation	17), 18)
								SM	phosphorylation	17)
P. aeruginosa	Pa-16	A 20718 (strain 130)	6.3	50	>100	50	>100	GM	3-acetylation	19)

*KM=kanamycin, SM=streptomycin, NM=neomycin, GM=gentamicin C, DKB=3′,4′-dideoxy-kanamycin B

* 3 % glycerol, 0.3 % sodium L-glutamate, 0.2 % peptone, 0.31 % Na_2HPO_4, 0.1 % KH_2PO_4, 0.005 % ammonium citrate, 0.001 % $MgSO_4$, 1.5 % agar.

greater activity.

The most interesting feature of BB-K 8 is its remarkable activity against a number of kanamycin-resistant organisms including those which are resistant because they can enzymatically inactivate aminoglycoside antibiotics. Table 6 summarizes the activity of BB-K 8 against the aminoglycoside-resistant organisms along with their reported mechanism of inactivation, indicating that BB-K 8 is neither inactivated by kanamycin 3′-phosphorylase, kanamycin 6′-acetylase, gentamicin 3-acetylase nor gentamicin adenylase.

The determination of the MIC against tubercle bacilli was made after 3 weeks incubation at 37°C, and the MIC data shown in Table 3 are geometric means of four independent tests. BB-K 8 was on the average about two to four-fold more active than kanamycin A against the kanamycin-sensitive strains though cross-resistance was apparent between the two antibiotics with the *Mycobacterium* species.

In Vivo Activity

BB-K 8 was evaluated *in vivo* comparatively with kanamycin in experimental infections in mice. The pathogenic bacteria employed were 2 strains of *S. aureus*, 3 strains of *E. coli* and 3 strains of *P. aeruginosa*, including both kanamycin-sensitive and resistant organisms. Mice were challenged intraperitoneally with $100 \times LD_{50}$ dose of the pathogens in a 5% suspension of hog gastric mucin. The antibiotics were administered subcutaneously just after the bacterial challenge on a single treatment schedule. The mice were observed for 5 days and the median curative dose (CD_{50}) was determined by the log-probit method[8].

Table 7. Chemotherapeutic activity of BB-K 8 and kanamycin in experimental infections of mice

Organism	Code	MIC (mcg/ml)		CD_{50} (mg/kg)	
		BB-K 8	Kanamycin	BB-K 8	Kanamycin
S.aureus Smith	Sa-2	0.4	0.4	2.3	2.2
S.aureus A 20239	Sa-10	1.6	100	7.2	74
E.coli NIHJ	Ec-1	0.4	0.8	2.2	4.2
E.coli Juhl	Ec-3	0.8	1.6	6.8	10.5
E.coli A 20363	Ec-5	0.8	>100	5.4	>400
P.aeruginosa D 15	Pa-1	1.6	25	8.6	68
P.aeruginosa H 9	Pa-4	6.3	>100	34	>400
P.aeruginosa A 15194	Pa-6	3.1	50	21	280

Table 8. Blood levels of BB-K 8 and kanamycin in mice

Antibiotic	Dose(sc)	Average blood levels (mcg/ml)			
		10 min.	30 min.	60 min.	120 min.
BB-K 8	40 mg/kg	48.7	47.6	12.8	4.2
	20	16.7	13.1	5.9	0.53
	10	9.7	7.8	4.4	0.43
Kanamycin	40 mg/kg	27.3	25.0	15.2	2.5
	20	12.0	11.2	6.6	0.43
	10	7.2	6.2	3.6	0.33

The results of the *in vivo* tests are summarized in Table 7 along with the *in vitro* MIC against the challenge organisms. The *in vivo* activities of BB-K 8 and kanamycin showed fair agreement with the *in vitro* data, and BB-K 8 afforded excellent protections in mice

Table 9. Urinary recovery of BB-K 8 and kanamycin in rats

Antibiotic	Dose	Total dose to 5 rats	Urine volume	Antibiotic level	Antibiotic recovered	Percent excretion
BB-K 8	20 mg/kg	21.4 mg	29 ml	570 mcg/ml	16.53 mg	77.2 %
Kanamycin	〃	22.4	26	600	15.60	69.6

against infections with the kanamycin-resistant organisms where kanamycin showed little or no effect.

Absorption, Excretion and Toxicity

Preliminary laboratory experiments on the absorption and excretion of BB-K 8 were carried out in mice and rats. Groups of 4 mice were administered graded dose (40, 20 and 10 mg/kg) of BB-K 8 and kanamycin subcutaneously. Blood samples were collected from orbital sinuses at 10, 30, 60 and 120 minutes post-administration and assayed by the paper disc agar diffusion method on *B. subtilis* plates.

The results of three independent experiments (total 12 mice/dose) are averaged and shown in Table 8. These data indicate that BB-K 8 is well absorbed in mice after subcutaneous administration with peak levels somewhat higher than those obtained with kanamycin, especially in mice receiving the 40 mg/kg/dose.

The urinary reeovery of BB-K 8 was studied in rats. Groups of 5 rats weighing 220~240 g were administered 20 mg/kg of BB-K 8 and kanamycin subcutaneously. They were placed in metabolism cages and the urine samples were collected during 0 to 8 hours post-administration. Antibiotic levels in urine samples were determined as described for the blood level test in mice, and the percent recovery was calculated from the antibiotic level, the urine volume and the total dose administered.

The results are summarized in Table 9, which shows high urine levels and high percentage excretion of BB-K 8 and kanamycin following parenteral administration. In view of the possible metabolism of BB-K 8 *in vivo*, the recovered urine specimens were examined by silica gel plate (Kieselgel F-254) thin-layer chromatography using solvent system S-110*. Bio-autography of the plates overlayed with *B. subtilis* indicated that intact BB-K 8 was the only bioactive substance in the urine sample. With kanamycin, unchanged antibiotic was also recovered.

The acute intravenous toxicity of BB-K 8 was determined on mice comparatively with kanamycin. Groups of 6 mice/dose (weighing 18~20 g) were used and observed for 4 days for the LD_{50} determination. Since it is known that the acute toxicity of aminoglycoside antibiotics varies considerably depending on the pH of the antibiotic solution[9], the LD_{50}s were determined at two pH levels. At pH 6.6, the intravenous LD_{50}s of BB-K 8 and kanamycin were found to be 340 (315~365) mg/kg and 280 (262~298) mg/kg, respectively, and at pH 7.4 the LD_{50}s were 560 (495~625) mg/kg and 430 (361~499) mg/kg, respectively. These data suggested that BB-K 8 might be somewhat less toxic than kanamycin.

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* Chloroform - methanol - 28 % ammonia - water (1 : 4 : 2 : 1).
Rf : BB-K 8 0.15~0.18, kanamycin 0.38~0.45

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