AMINOGLYCOSIDE ANTIBIOTICS. V

THE 4′-DEOXYBUTIROSINS (BU-1975C_1 AND C_2), NEW AMINOGLYCOSIDE ANTIBIOTICS OF BACTERIAL ORIGIN

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Strains of *Bacillus circulans* produced the new aminoglycoside antibiotics, 4′-deoxybutirosins A and B (Bu-1975C_1 and C_2). The new antibiotics, $C_{21}H_{41}N_5O_{11}$, have physicochemical properties similar to butirosins A and B ($C_{21}H_{41}N_5O_{12}$) with specific differences in TLC and NMR. The antibacterial activity and spectra of 4′-deoxybutirosins are similar to but broader than that of butirosins, inhibiting some butirosin-resistant organisms and showing increased anti-pseudomonal activity. The acute intraveous toxicity of Bu-1975C_1 is lower than that of kanamycin and similar to butirosin A.

An antibiotic complex, Bu-1975, has been isolated from fermentation broth of three strains of *Bacillus circulans*. The antibiotic is a complex of at least five bio-active components, A_1, A_2, B, C_1 and C_2. Components A_1 and A_2 were identified as butirosins A and B[1,2], respectively, and component B was a solvent-extractable antibiotic with peptide-like properties. Components C_1 and C_2 are new aminoglycoside antibiotics which appeared to be closely related to butirosins A and B. As reported in a companion paper[3], Bu-1975C_1 is composed of D-xylose, 2-deoxystreptamine, L-(−)-γ-amino-α-hydroxybutyric acid and 2,6-diamino-2, 4, 6-trideoxy-D-*xylo*-hexopyranose, a new deoxy amino sugar. Its structure has been determined to be 4′-deoxybutirosin A. Component C_2 contains D-ribose in place of D-xylose of component C_1 and hence it is 4′-deoxybutirosin B. Bu-1975C_1 and C_2 exhibit a broader spectrum of antibacterial activity than kanamycin and butirosin inhibiting some kanamycin and/or butirosin-resistant organisms. This paper reports on the producing organism, fermentation, isolation and the physico-chemical and biological properties of Bu-1975C_1 and C_2.

Producing Organisms

Three bacterial strains have been found to produce the antibiotic complex Bu-1975, which were designated as strain Nos. C308-B4, C436-B1 and C532-B2 in our culture collection. The morphological and cultural characteristics of these strains are shown in Table 1. The physiological reactions and carbon source utilization of the strains are shown in Tables 2 and 3, respectively.

In view of the morphological, cultural and physiological characteristics described in these tables, all of the three strains are believed to belong to the species *Bacillus circulans* although there are certain differences from the description of the type species of *Bacillus circulans*.

Table 1. Morphological and cultural characteristics of Bu-1975 producing organisms.

	C308-B4	C436-B1	C532-B2
Vegetative cells:	Rods, 0.4~0.6×1.5~4.0 μ, with ends rounded, not in chains. Some palisade arrangement. Gram-variable, mostly negative.	Rods, 0.6~0.8×2.0~6.0 μ, with ends rounded, not in chains. Gram-variable, mostly negative.	Rods, 0.4~0.6×1.0~5.0 μ, with ends rounded, not in chains. Gram-variable, mostly negative.
Spores:	Oval to ellipsoidal, 0.9~1.4×1.4~2.2 μ; terminal to subterminal, spore-wall thick and easily stained.	Oval to ellipsoidal, 0.9~1.2×1.3~2.2 μ; paracentral to terminal, spore-wall thick and easily stained.	Oval to ellipsoidal, 1.0~1.4×1.5~2.4 μ; terminal, spore-wall thick and easily stained.
Sporangia:	Definitely swollen and racket-shaped.	Definitely swollen and spindle to racket-shaped.	Definitely swollen and racket-shaped.
Motility:	Motile cell not found.	Motile	Motile
Nutrient agar slant:	Growth abundant, thick, opaque, smooth, viscous and creamy becoming pale-orange with age.	Growth late, moderate, thin, opaque, smooth, viscous and creamy.	Growth abundant, thick, opaque, smooth, slightly viscous and whitish.
Glucose asparagine agar slant:	Moderate growth. Raised, semi-translucent, smooth, glistening, wrinkled, viscid and slight-yellow.	Scant growth. Thin, translucent, smooth, viscid and colorless.	No or scant growth.
Glucose broth:	pH 5.3~5.0 (37°C). Lightly viscous pellicle or ring growth. Produced turbidity and sediment.	pH 7.5~8.0 (28°C). Viscous pellicle. Light turbidity and sediment.	pH 6.8~5.8 (37°C). No pellicle. Light turbidity and heavy sediment. Glucose broth plus biotin: pH 8.0~9.0. Complete pellicle. Turbidity and light sediment.
Colony on nutrient agar:	Circular, raised to domed with entire margin. Opaque with smooth and glistening surface. Size: 1.0~3.5 mm (in dia.). Viscous and pale-orange. No satellite colony.	Circular or slightly irregular, raised with irregular margin. Opaque with smooth surface. Size: 1.0~4.0 mm. (in dia.). Viscous and creamy. No satellite colony.	Flat to a little raised with slightly irregular margin. Opaque with smooth surface. Size: 0.8~4.0 mm (in dia.). Slightly viscous and creamy white. No satellite colony.
Growth temperature			
Optimum:	37°C	30°C	45°C
Moderate growth:	28~45°C	22~37°C	28~50°C
Restricted growth:	20°C, 48°C	20°C, 40°C	25°C, 53°C
Scant growth:	18°C, 50°C	18°C, 42°C	20°C, 55°C
No growth:	15°C, 53°C	12°C, 45°C	15°C, 58°C
Oxygen demand:	Aerobic	Aerobic	Aerobic
NaCl broth			
Growth:	0~1 % NaCl	0~3 % NaCl	0~2 % NaCl
No growth:	2 % NaCl	4 % NaCl	3 % NaCl
Milk:	Viscous, thick, pale orange pellicle with viscous sediment. No other change.	Viscous, thick pellicle with viscous sediment. No other change.	Viscous, thick, faint-orange pellicle with scant sediment. Coagulated without peptonization. pH slightly alkalized.
Gelatin stab:	Viscid, pale-orange pellicle. Liquefied gelatin completely after 1 week.	Viscid, creamy pellicle. Liquefied gelatin completely after 1 week.	Viscid, whitish pellicle. Liquefied gelatin completely after 1 week.
Growth factor:	Nicotinic acid, biotin and complex of amino acids are accelerative for growth.	Nicotinic acid, biotin and complex of amino acids are accelerative for growth.	Biotin is essential for growth.

Table 2. Physiological reactions.

Test	C308-B4	C436-B1	C532-B2
Gas from carbohydrates	Negative	Negative	Negative
Gelatin liquefaction	Positive (strong)	Positive (strong)	Positive (strong)
Indole production	Negative	Negative	Negative
Starch hydrolysis	Positive	Positive	Positive
(Iodine reaction's color)	(orange yellow)	(orange yellow)	(orange yellow)
Citrate utilization	Negative	Negative	Negative
Nitrite from nitrate	Positive	Positive	Positive
Urease reaction	Negative	Negative	Negative
Catalase reaction	Positive	Positive	Positive
Oxidase reaction	Negative	Negative	Negative

Table 3. Acid production from carbohydrates.

	C308-B4			C436-B1			C532-B2		
	I*	II	III	I	II	III	I	II	III
Glycerol	+	+	++	+	±	+	+	+	++
L-Arabinose	−	−	−	+	−	−	−	−	−
D-Xylose	−	−	−	+	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−	−	−	−
D-Fructose	+	+	−	−	−	−	+	−	−
D-Galactose	+	+	++	+	+	+	+	+	−
D-Glucose	+	+	++	+	+	++	+	+	++
D-Mannose	+	+	−	+	−	−	+	±	−
Sucrose	+	+	+	+	+	++	+	+	+
Lactose	+	+	+	−	−	−	+	±	−
Maltose	+	+	++	+	+	++	+	+	++
D-Raffinose	±	−	−	−	−	−	±	−	−
Inositol	+	+	+	±	−	−	+	+	±
D-Mannitol	−	−	−	−	−	−	−	−	−
D-Sorbitol	−	−	−	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−	−
Starch	+	+	++	+	+	+	+	+	++
Cellulose	−	−	−	−	−	−	−	−	−
Inuline	−	−	−	−	−	−	−	−	−
Salicine	+	+	+	+	+	−	+	+	±

* Basal medium I: 0.1 % NH_4NO_3, 0.1 % K_2HPO_4, 0.03 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.01 % $CaCl_2 \cdot 2H_2O$, 0.05 % NaCl with 10 mcg/ml each of aspartate, glutamate and tryptophane, 1 mcg /ml of nicotinic acid, 0.05 mcg/ml of biotin.
Basal medium II: 0.1 % NH_4Cl; 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % NaCl, 0.1 % $CaCO_3$, 0.01 % yeast extract, 1.5 % Bacto-agar with 1 mcg/ml of nicotinic acid, 0.05 mcg/ml of biotin
Basal medium III: Peptone broth.

Significant differences of each of the three strains from the typical *B. circulans* are summarized below:

Strain C308-B4

Color of colony: Pale orange.

Growth factor: Nicotinic acid, biotin and amino acids complex accelerate the growth.
Sugar utilization: L-Arabinose and D-xylose are not utilized.

Strain C436-B1

Glucose broth: Weakly alkaline reaction (pH 7.5~8.0) after 3~5 days.
Growth temperature: Lower optimum temperature (30°C) and no growth at 45°C.
Growth factor: Nicotinic acid, biotin and amino acids complex accelerate the growth.

Strain C532-B2

Growth factor: Biotin is essential for the growth.
Sugar utilization: L-Arabinose and D-xylose are not utilized.

In view of the above differences, the following subspecies names are proposed for the three strains of Bu-1975 producing organism.

Bacillus circulans subsp. n. *croceus* (strain C308-B4).

Bacillus circulans subsp. n. *proteophilus* (strain C436-B1).

Bacillus circulans subsp. n. *biotinicus* (strain C532-B2).

Antibiotic Production

A well-grown agar slant of the Bu-1975-producing organism was used to inoculate seed medium containing 1.5% glucose, 0.5% Polypeptone, 0.2% yeast extract, 0.05% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, the pH being adjusted to 7.5 before sterilization. The seed culture was incubated at 37°C for 24 hours on a rotary shaker (250 rpm), and 2 ml of the growth was transferred to 100 ml of the 3% soybean meal, 2% corn starch, 1% $CaCO_3$ and 0.33% $MgSO_4 \cdot 7H_2O$ fermentation medium in a 500-ml Erlenmeyer flask. Antibiotic production reached a maximum after 3~6 days shaking at 28°C.

The antibiotic activity in the fermentation broth was determined by a paper disc-agar diffusion assay using *Bacillus subtilis* PCI 219 and *Klebsiella pneumoniae* A20680.* All components of the Bu-1975 complex (A_1, A_2, B, C_1 and C_2) showed activity against *B. subtilis* PCI 219 but only two components, C_1 and C_2, were active against *K. pneumoniae* A20680.

The production of the C components relative to the others was different with different strains. In the shake flask fermentation, strain C532-B2 produced 200~250 mcg/ml of the C components which represented about 30~50% of the total bio-activity assayed by *B. subtilis*. Other strains were less productive of the C components than strain C532-B2.

Isolation and Purification

The harvested broth was filtered with filter aid and the bio-activity in the filtrate (pH 8.0) was adsorbed by a column of Amberlite IRC-50 (NH_4^+ form). The column was washed with water and then developed with 1 N NH_4OH. The active eluates were combined, concentrated *in vacuo* and extracted with *n*-butanol to remove component B from the concentrate. The aqueous layer was separated and applied to a column of Amberlite CG-50 (NH_4^+). The column was washed with water and N/4 NH_4OH successively, and the activity was eluted with N/2

* Original strain is *K. pneumoniae* Type 22 #3038 which is known to produce neomycin-kanamycin phosphotransferase II and gentamicin adenylate synthetase.

Table 4. TLC of Bu-1975 components.

System	Plate	Solvent system	Rf			
			A_1*	A_2	C_1	C_2
S-110**	Silica gel	$CHCl_3$-MeOH-28 % NH_4OH-H_2O (1:4:2:1)	0.41	0.41	0.51	0.51
S-117	Silica gel	$CHCl_3$-MeOH-28 % NH_4OH (1:3:2)	0.20	0.20	0.26	0.26
S-115***	Alumina	upper phase of $CHCl_3$-MeOH-17 % NH_4OH (2:1:1)	3.7 cm	0.2 cm	5.3 cm	0.5 cm

* A_1=butirosin A, A_2=butirosin B.
** developed three times succesively
*** 16 hours development, location is shown in cm from the origin

Fig. 1. IR spectrum of Bu-1975C_1.

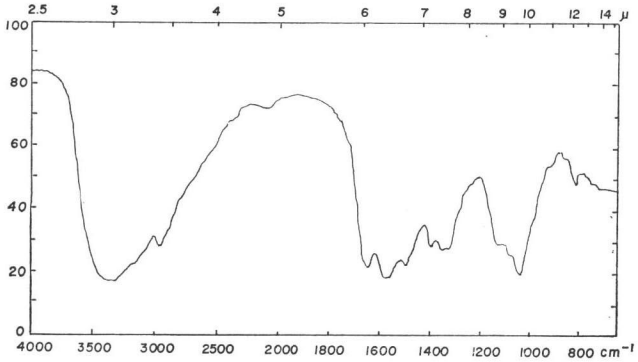
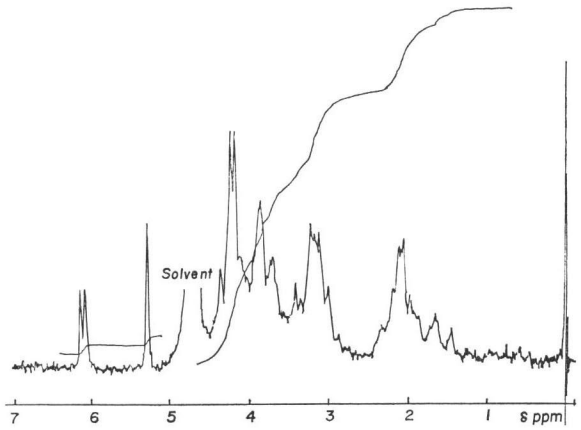


Fig. 2. NMR spectrum of Bu-1975C_1·HCl (60 MHz, in D_2O).



NH_4OH, the eluate being collected fractionally. The active components were eluted in the order of A_2, A_1, C_2 and C_1, though with considerable overlap. Complete separation of each component was achieved by repeating the CG-50 column chromatography.

As shown in Table 4, two TLC systems, S-110 and S-117, were found suitable to differentiate components A_1 and A_2 from components C_1 and C_2, and system S-115, when developed for 16 hours, gave a separation of A_1 from A_2 and C_1 from C_2.

Components A_1 and A_2 were identified as butirosins A and B[1], respectively, by physico-chemical properties (TLC, IR and NMR) and antibacterial spectrum. The following concerns the unique components C_1 and C_2 of antibiotic Bu-1975.

Physico-chemical Properties of Bu-1975C_1 and C_2

Bu-1975C_1 and C_2 are white amorphous solid bases, which are readily soluble in water, slightly soluble in methanol and ethanol, and practically insoluble in *n*-butanol, acetone and other organic solvents. Both components give positive reactions with ninhydrin and anthrone reagents but are negative in TOLLENS, FEHLING and SAKAGUCHI reactions.

An analytical sample of Bu-1975C_1 was isolated in a form of crystalline monosulfate, which melted at 245 ~ 247°C (decomp.), $[\alpha]_D^{21}$ +24.5°(*c* 1.0, water), and analyzed for $C_{21}H_{41}N_5O_{11}$ ·H_2SO_4· 3/2 H_2O.

Anal. Calc'd: C 37.94, H 6.98, N 10.54, S 4.82.
Found: C 37.96, H 7.41, N 10.00, S 4.75.

It gave a tetra-N-acetate, mp>250°C, $[\alpha]_D^{24.5}$+26.0° (*c* 0.5, water), which analyzed for $C_{21}H_{41}N_5O_{11}\cdot(C_2H_2O)_4\cdot 3/2\,H_2O$.

Anal. Calc'd: C 47.40, H 7.13, N 9.53.
Found: C 47.30, H 7.47, N 9.65.

An analytical sample of Bu-1975C_2 was obtained as dicarbonate, which melted at 172~178°C (decomp.), $[\alpha]_D^{25}$+30.0° (*c* 1.0, water), and analyzed for $C_{21}H_{41}N_5O_{11}\cdot 2H_2CO_3$.

Anal. Calc'd: C 41.63, H 6.83, N 10.55.
Found: C 41.22, H 6.67, N 10.82.

The tetra-N-acetate of Bu-1975C_2 was also prepared, mp>250°C, $[\alpha]_D^{24.5}$+43° (*c* 0.5, water), and analyzed for $C_{21}H_{41}N_5O_{11}\cdot(C_2H_2O)_4\cdot H_2O$.

Anal. Calc'd: C 47.99, H 7.08, N 9.65.
Found: C 48.09, H 7.20, N 9.42.

Bu-1975C_1 and C_2 show end absorption only in the UV region. The IR spectra of Bu-1975C_1 (Fig. 1) and C_2 are nearly identical, which are also quite similar to those of the butirosins. The NMR spectrum of Bu-1975C_1 (Fig. 2) shows two anomeric protons at δ 5.28(s) and 6.10 (d, J=3.5 Hz) ppm, the lowerfield signal being different from that of Bu-1975C_2 which shows the anomeric protons at δ 5.28 (s) and 5.98 (d, J=3.5 Hz) ppm. A similar difference in the chemical shift of the second anomeric proton was seen in the NMR spectra of butirosins A and B.[22] The comparative NMR data of Bu-1975C_1 and C_2 along with butirosins A and B are shown in Table 5. As also shown in the table, the ratio of proton integrals for the higher (δ 1.2~2.4 ppm) and the lower (δ 2.5~4.4 ppm) methylene-methine regions is 6:18 for Bu-1975C_1 and C_2 in contrast to a ratio of 4:19 for butirosins A and B.

Table 5. NMR data for Bu-1975C_1 and C_2 (4′-deoxybutirosins A and B) and butirosins A and B (60 MHz, in D_2O pH 2.0)

Chemical shift (δ, ppm)	Number of protons and type of signals			
	Bu-1975C_1	Bu-1975C_2	Butirosin A	Butirosin B
1.2~2.4	6H (m)	6H (m)	4H (m)	4H (m)
2.5~4.4	18H (m)	18H (m)	19H (m)	19H (m)
5.16	—	—	1H (s)	1H (s)
5.28	1H (s)	1H (s)	—	—
5.98	—	1H(d, J=3.5 Hz)	—	1H (d, J=3.6 Hz)
6.10	1H(d, J=3.5 Hz)	—	1H (d, J=3.6 Hz)	—

Biological Activity

Antibacterial Spectrum

The minimum inhibitory concentrations (MIC) of Bu-1975C_1 and C_2 were determined against a wide variety of bacteria by the two-fold agar dilution method on Nutrient Agar (Eiken) plates using the Steer's multi-inoculating apparatus. Inoculum was standardized to be a 10^4 dilution of overnight culture of the test organism in Heart Infusion Broth (Difco).

Table 6. Antibacterial spectra of Bu-1975C_1 and C_2.

Test organisms	MIC (mcg/ml)			
	Bu-1975C_1	Bu-1975C_2	Kanamycin	Butirosin A
Staphylococcus aureus Smith	0.4	0.8	0.2	0.4
〃 〃 209P	3.1	3.1	0.8	3.1
〃 〃 A20239	1.6	1.6	50	6.3
Escherichia coli NIHJ	0.8	0.8	0.8	0.8
〃 〃 Juhl	0.8	0.8	0.8	0.8
〃 〃 ML-1630	0.8	0.8	>100	0.8
〃 〃 A20365	0.2	0.2	100	0.2
〃 〃 K12	0.4	0.8	0.8	0.4
〃 〃 NR79/W677	0.8	3.1	3.1	0.8
〃 〃 JR35/C600	0.4	0.8	100	0.4
〃 〃 W677	0.8	0.8	0.4	0.8
〃 〃 JR66/W677	0.8	1.6	100	25
Klebsiella pneumoniae D11	0.1	0.2	0.2	0.2
〃 〃 Type 22, #3038	0.8	1.6	100	50
Proteus vulgaris A9436	0.2	0.2	0.4	0.1
〃 〃 A9716	1.6	1.6	0.8	1.6
Proteus morganii A9553	0.4	0.4	0.8	1.6
〃 〃 A20031	0.8	0.8	3.1	0.8
〃 〃 A15153	1.6	1.6	1.6	1.6
〃 〃 A15166	0.4	0.4	0.2	0.4
Proteus mirabilis A9554	0.8	0.8	0.4	1.6
〃 〃 A9900	0.4	0.8	0.4	1.6
Serratia marcescens A20019	1.6	6.3	0.8	6.3
Pseudomonas aeruginosa D15	3.1	3.1	25	6.3
〃 〃 A9923	6.3	6.3	50	12.5
〃 〃 A9930	0.4	0.4	12.5	1.6
〃 〃 H9	25	25	>100	>100
〃 〃 A20479	6.3	12.5	>100	12.5
〃 〃 A20653	100	25	>100	>100
〃 〃 A9843	6.3	3.1	100	25
Pseudomonas sp. A20355	3.1	3.1	50	6.3
〃 A20603	25	25	>100	100
〃 A20618	>100	>100	>100	>100
Mycobacterium 607	0.4	0.4	0.4	0.4
Mycobacterium phlei	0.2	0.2	0.4	0.2
Mycobacterium ranae	0.4	0.4	0.4	0.4

Mycobacterium strains were tested on plates containing No. 1001 agar medium*, the inoculum being a 10^3 dilution of the overnight culture. The results are shown in Table 6 along with those for butirosin A and kanamycin which were tested for comparison. Bu-1975C_1 and C_2 have essentially the same level of intrinsic potency. They exhibit a broad spectrum of antibacterial activity against gram-positive and gram-negative bacteria including those which are

* 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.

resistant to the commonly used aminoglycoside antibiotics such as streptomycin, neomycin, kanamycin and gentamicin. In general, the activity and spectrum of Bu-1975C_1 and C_2 are similar to those of butirosin. However, Bu-1975C_1 and C_2 have a broader spectrum than butirosin inhibiting some butirosin-resistant organisms, and showing somewhat greater anti-pseudomonas activity than butirosin.

The activity of Bu-1975C_1 was compared with reference aminoglycoside antibiotics in MUELLER-HINTON medium (Difco) against various strains of aminoglycoside-resistant organisms whose mechanisms of resistance have been reported. It can be seen from the results shown in Table 7 that Bu-1975C_1 is resistant to various types of the aminoglycoside-inactivating enzymes. Like butirosin, it is refractory to the actions of neomycin-kanamycin phosphotransferase I (NPT_1) and gentamicin adenylate synthetase (GAS). Moreover Bu-1975C_1 was active against strains of the neomycin-kanamycin phosphotransferase II (NPT_2)-producing organisms (Ec-49, Ec-53, Kp-8 and El-12 in Table 7) which are known to inactivate butirosin by the 3′-phosphorylation. Both Bu-1975C_1 and butirosin showed reduced activity against the strains

Table 7. Activity of Bu-1975C_1 against aminoglycoside-resistant organisms.

Organisms	Code#	MIC (mcg/ml) in MUELLER-HINTON medium					Inactivating enzyme*	Reference
		Bu-1975C_1	Butirosin A	Kanamycin A	Neomycin B	Gentamicin C		
S. aureus Smith	Sa-2	1.6	1.6	0.8	1.6	0.4	–	
" " A20239	Sa-10	6.3	25	>100	>100	1.6	NPT_1+NPT_2	23
E. coli NIHJ	Ec-1	1.6	1.6	1.6	3.1	0.8	–	
" " ML-1630	Ec-5	6.3	6.3	>100	>100	3.1	NPT_1	6
" " NR79/W677	Ec-9	12.5	12.5	25	6.3	1.6	KAT	7,15
" " JR35/C600	Ec-10	1.6	1.6	>100	>100	1.6	NPT_1	11
" " A20107	Ec-49	6.3	100	>100	100	3.1	NPT_2	23
" " JR66/W677	Ec-53	3.1	>100	>100	>100	50	NPT_2+GAS	13,14,19
" " R5	Ec-55	25	25	50	6.3	1.6	KAT	5
" " A20895	Ec-62	3.1	3.1	3.1	6.3	100	GAT_1	23
" " A20732	Ec-72	3.1	3.1	50	6.3	100	GAS	23
K. pneumoniae 22-3038	Kp-8	3.1	>100	>100	>100	100	NPT_2+GAS	18
E. cloacae A21006	El-12	6.3	>100	>100	>100	3.1	NPT_2	23
P. aeruginosa A9930	Pa-3	3.1	6.3	50	25	1.6	–	
" " strain 130	Pa-16	25	50	>100	>100	>100	GAT_1+NPT	8,23
" " GN 315	Pa-27	>100	>100	>100	>100	25	KAT	24,25
P. stuartii A20894	Ps-2	>100	>100	3.1	>100	50	GAT_2	23

* NPT_1: neomycin-kanamycin 3′-phosphotransferase I
NPT_2: neomycin-kanamycin-butirosin 3′-phosphotransferase II
KAT: kanamycin 6′-acetyltransferase
GAS: gentamicin-DKB 2″-adenylate synthetase
GAT_1: gentamicin 3-acetyltransferase I
GAT_2: gentamicin 2′-acetyltransferase II

which are known to produce kanamycin 6′-acetyltransferase (KAT) and gentamicin 2′-acetyltransferase II (GAT_2).

In Vivo Activity and Toxicity

Bu-1975C_1 was evaluated *in vivo* comparatively with butirosin A in experimental infections

of mice. The pathogenic bacteria employed were *Staphylococcus aureus* Smith, *Escherichia coli* NIHJ and *K. pneumoniae* A20680, a kanamycin- and butirosin-resistant organism. Mice were challenged intraperitoneally with a $100\times LD_{50}$ dose of the pathogens in a 5% suspension of hog gastric mucin. A single subcutaneous treatment with the antibiotic was given immediately after the bacterial challenge. Group of 5 mice was used for each dosage level and the animals

Table 8. *In vivo* activity of Bu-1975C_1.

Dose (sc) mg/kg	*S. aureus* Smith infection		*E. coli* NIHJ infection		*K. pneumoniae* A20680 infection	
	Bu-1975C_1	Butirosin A	Bu-1975C_1	Butirosin A	Bu-1975C_1	Butirosin A
100					—	1/5
50					—	1/5
25	5/5*	5/5	5/5	5/5	5/5	0/5
12.5	5/5	5/5	5/5	5/5	3/5	0/5
6.25	4/5	4/5	4/5	3/5	2/5	0/5
3.12	3/5	3/5	2/5	3/5	2/5	0/5
1.56	2/5	1/5	0/5	1/5	1/5	0/5
PD_{50}	2.5 mg/kg	2.9 mg/kg	3.8 mg/kg	3.7 mg/kg	6.3 mg/kg	>100 mg/kg

* No. survived/No. infected
** Determined by the method of J.L. REED *et al.*[4]

were observed for 5 days to determine the median protective dose (PD_{50}).

The results of the *in vivo* experiments are shown in Table 8. Bu-1975C_1 afforded excellent protection in mice against all three of the infections tested. Butirosin A showed activity *in vivo* comparable to Bu-1975C_1 against the sensitive *S. aureus* and *E. coli* infections but was inactive against the resistant *K. pneumoniae* infection.

The acute toxicity of Bu-1975C_1 was determined in mice, and the LD_{50} and ranges were computed by the method of MILLER and TAINTER.[26] The intravenous LD_{50}* was 520 (515~526) mg/kg for the sulfate of Bu-1975C_1 and greater than 1,000 mg/kg for the free base form. In a comparative experiment the intravenous LD_{50}s of kanamycin sulfate and butirosin sulfate were found to be 280 (272~288) mg/kg and 520 (502~538) mg/kg, respectively.

Discussion

In recent years there has been remarkable progress in elucidating the mechanisms of R-factor mediated resistance to the aminoglycoside antibiotics. These resistant organisms are now known to produce bacterial enzymes which inactivate aminoglycoside antibiotics by acetylation,[5,7,8,24,25,27] phosphorylation[6,9,10,11,19] and adenylylation[12,13,14]. Neomycin-kanamycin phosphotransferase I is known to inactivate kanamycin, neomycin, paromomycin and ribostamycin by the 3′-phosphorylation[15,21]. Recently it has been reported[20,21] that the same enzyme phosphorylates the 5″-OH of lividomycin which lacks in the 3′-OH. Gentamicins C_1, C_2 and C_{1a}, tobramycin[16] and dideoxykanamycin B[17], which have no hydroxyl group in the 3′-position, are incapable of the phosphorylative inactivation. Butirosin is not inactivated by neomycin-kanamycin phosphotransferase I though it has a 3′-hydroxyl group, and the resistance of

* LD_{50} and ranges are expressed as weight of the free base.

butirosin to this enzymatic inactivation is supposed to be due to the acyl substitution of 1-amino function with L(−)-γ-amino-α-hydroxybutyric acid. This is also the case with Bu-1975C_1 and C_2 (4′-deoxybutirosins), which showed activity against the kanamycin-resistant organisms that produce neomycin-kanamycin phosphotransferase I.

Of particular interest is the fact that Bu-1975C_1 and C_2 inhibit *Enterobacter cloacae* A20106, *E. coli* A20107, *E. coli* JR66/W677 and *K. pneumoniae* type 22 #3038, all of which produce neomycin-kanamycin phosphotransferase II and inactivate butirosin by 3′-phosphorylation[10]. The latter two organisms are known to co-produce gentamicin adenylate synthetase which inactivates gentamicin C components[13] and 3′, 4′-dideoxykanamycin B[14] by 2″-adenylylation. The increased anti-pseudomonal activity of Bu-1975C_1 and C_2 over butirosins may be explained by an assumption that a substantial number of *Pseudomonas* strains are producing or co-producing the neomycin-kanamycin phosphotransferase II along with other types of inactivating enzymes. As mentioned before and described in detail in a succeeding paper[3], Bu-1975C components differ from butirosins only in the lack of hydroxyl group at the 4′-position. The role of the 4′-hydroxyl group in aminoglycoside antibiotics has not been studied with respect to the inactivation mechanisms of resistant organisms carrying R-factor, and it is hoped that the activity of 4′-deoxybutirosins may serve as a lead to further clarification of aminoglycoside inactivation mechanisms.

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