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Butirosin, a New Aminoglycosidic Antibiotic Complex: Bacterial Origin and Some Microbiological Studies

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Butirosin, a new aminoglycosidic antibiotic complex, was produced by submerged fermentation with each of two strains of *Bacillus circulans*. A paper-disc, agar-diffusion assay which employs *Escherichia coli* (P-D 04863) as the test organism has been developed. Shaken-flask and stirred-jar fermentations in a medium containing glycerol, soybean meal, meat peptone, ammonium chloride, and calcium carbonate reach titers of 500 to 700 μg of butirosin base per ml. Butirosin is active against several gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*.

Butirosin (P. W. Woo, G. L. Coffey, H. W. Dion, S. A. Fusari, and G. D. Senos, U.S. Pat. no. 3,541,078.) is a new, relatively nontoxic antibiotic complex active in vitro and in vivo against various pathogenic gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa* (3). The complex has been resolved into two, white, amorphous, water-soluble bases, butirosin A (80 to 85%) and butirosin B (15 to 20%), isomers which differ only in the configuration at one carbon atom in the pentose moiety. Butirosin A is *N'*-[(S)-(−)-4-amino-2-hydroxybutyryl]-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-β-D-xylofuranosyl-2-deoxystreptamine, also known in the literature (Woo et al, U.S. Pat. no. 3,541,078) as *N'*-(4-amino-2-hydroxybutyryl)-4-*O*-(2,6-diamino-2,6-dideoxy-D-glucopyranosyl)-5-*O*-D-xylofuranosyl-2-deoxystreptamine; butirosin B contains D-ribose in place of D-xylose (8–10). The antibiotic complex was obtained (1) from fermentation filtrates of each of two strains of *Bacillus circulans*, a novel source for a water-soluble, basic antibiotic.

MATERIALS AND METHODS

Producing cultures. Strain 1 was isolated from a soil sample collected near Nelspruit, East Transvaal, South Africa. Strain 2 was obtained as a single colony isolate from strain 1. The two strains are similar, differing primarily in the amounts of butirosin produced.

Microbiological assay. Butirosin fermentation and fractionation samples, dosage forms, and clinical specimens are assayed by a paper-disc, agar-diffusion method similar to those employed for viomycin (2) but modified as follows. The test organism is a strain of *Escherichia coli* (P-D 04863), maintained on Nutrient Agar (Difco). The inoculum is prepared by growing the culture at 37 C for 18 hr in Veal Infusion Broth (Difco). The assay plates are prepared with a basal layer (2 ml/12.5 cm² surface area) of Mycin Assay Agar (Difco) plus 0.2% agar and a seed layer (1 ml/12.5 cm² surface area) of the same medium containing a 1,000-fold dilution of the broth culture. Incubation is at 37 C for 18 hr. Samples and reference standard are diluted in buffer composed of 0.1 M potassium phosphate solutions mixed in proportions giving a *p*H of 7.8. The reference standard is a sample of an amorphous butirosin sulfate salt containing about 71% butirosin base (80% butirosin A, 20% butirosin B). Standard concentrations of 20, 15, 10, 7.5, 5.0 (strong point), 4.0, 3.1, 2.0, 1.5, and 1.0 μg of butirosin base per ml give zones of inhibition typically 25.0, 24.2, 23.2, 22.4, 21.3, 20.6, 19.7, 18.3, 18.2, and 15.4 mm, respectively, in diameter.

Analytical procedures. Butirosin fermentation samples were analyzed for ammonia and glycerol by Auto-Analyzer procedures, ammonia by the published method of Logsdon (5), and glycerol by the chromotropic acid method (2) adapted to the AutoAnalyzer by C. Perrizo of our laboratories.

Bacterial growth in fermentation samples was determined turbidimetrically by measuring the light transmittance of 1:100 water dilutions in a Lumentron Colorimeter at 515 nm.

RESULTS AND DISCUSSION

Culture studies. The butirosin-producing organisms are aerobic, gram-variable, motile rods and form endospores in sporangia that are definitely swollen. The spores are ellipsoidal, rarely cy-

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TABLE 1. *Comparison of strain 2 with Bacillus circulans ATCC 4513*

Characteristic on medium	Strain 2	ATCC 4513
Morphology, etc., Nutrient Agar (Difco), 24-hr growth at 28 C; Hucker's modification of Gram stain	Gram-variable, usually single rods with rounded or pointed ends, 0.3 to 0.9 by 1.1 to 4.7 μm	Gram-variable, usually single rods with rounded ends, 0.3 to 1.0 by 2.0 to 6.5 μm
Peptone Iron Agar (Difco) plus 0.1% yeast extract (Difco), growth at 28 C; Conklin's spore stain	Good sporulation at 2 days; sporangia bulged and clavate; spores ellipsoidal, occasionally reniform, terminal and subterminal; spore wall thick; spores 0.6 to 1.3 by 1.1 to 2.3 μm	Good sporulation at 5 days; sporangia bulged and clavate; spores ellipsoidal, occasionally spherical, usually terminal; spore wall thin; spores 0.4 to 1.1 by 0.8 to 1.5 μm
Motility, Nutrient Broth (Difco), 24-hr growth at 28 C	Motile	Motile
Growth temperature, Nutrient Agar (Difco)		
28 C	Positive	Positive
37 C	Positive	Positive
45 C	Negative	Positive
65 C	Negative	Negative
Growth characteristics in broth		
Surface	None	None
Subsurface	Turbid	Turbid
Amount	Moderate	Moderate
Sediment	White flocculent	White flocculent
Colony characteristics, Nutrient Agar (Difco)		
Form	Circular, few punctiform	Irregular, few circular
Elevation	Convex	Flat
Margin	Entire	Undulate to erose
Surface	Smooth	Smooth
Density	Translucent	Translucent
Consistency	Viscid to butyrous	Butyrous
Color	Colorless to whitish	Colorless to whitish, slightly iridescent
Gelatin hydrolysis		
Nutrient Agar (Difco) plus 0.4% gelatin (Difco);	35- to 37-mm zone at 7 days	1- to 2-mm zone at 7 days
Gelatin (Difco) stab	Liquefaction at 5 days	No liquefaction at 5 and 13 days
Casein hydrolysis	Negative at 6 days Positive at 13 days	Negative at 6 and 13 days
Indole production	Negative at 5 and 13 days	Negative at 5 and 13 days
Reduction of nitrate to nitrite	Negative at 5 and 13 days	Negative at 5 and 13 days
Acetylmethylcarbinol production	Negative at 2, 6, and 13 days; *p*H of broth at 6 days, 5.8	Negative at 2, 6, and 13 days; *p*H of broth at 6 days, 5.8
Starch hydrolysis	Positive at 6 days, 1- to 2-mm zone	Positive at 6 days, 4- to 5-mm zone
Citrate utilization	Negative at 6 days Positive at 13 days	Negative at 6 and 13 days

TABLE 1—*Continued*

Characteristic on medium	Strain 2		ATCC 4513	
Methylene blue reduction	Positive at 2, 5, and 13 days		Positive at 2 days, reoxidized at 5 days	
Fermentation (28 C)	*Acid*	*Gas*	*Acid*	*Gas*
Ammonium-salts agar				
L-Arabinose	−	−	+	−
Dextrin	+	−	+	−
D-Fructose	+	−	+	−
D-Galactose	+	−	+	−
D-Glucose	+	−	+	−
Glycerol	+	−	+	−
Inulin	−	−	+	−
Lactose	−	−	+	−
Maltose	+	−	+	−
D-Mannitol	+	−	+	−
D-Mannose	+	−	+	−
Raffinose	+	−	+	−
Salicin	±	−	+	−
Sucrose	+	−	+	−
D-Xylose	−	−	+	−
Phenol Red Broth (Difco)				
L-Arabinose	−	−		
D-Glucose[a]	+	−	+	−
D-Mannitol	+	−		
Sucrose	+	−		

[a] *p*H after 6 days: strain 2, 5.5; ATCC 4513, 5.3.

lindrical, central to terminal, and have a thick, easily stained wall. The isolates are viscid on ordinary media, hydrolyze starch, do not produce gas from carbohydrates, do not produce indole or acetylmethylcarbinol, and do not grow at 65 C. The isolates are therefore regarded as strains of *B. circulans*.

Strain 2 was compared with *B. circulans* ATCC 4513, proposed neotype (7; see Table 1). The differences include size of vegetative cells and spores, thickness of spore wall, growth at 45 C, growth characteristics on nutrient agar, hydrolysis of gelatin and casein, citrate utilization, reoxidation of methylene blue, and fermentation of L-arabinose, inulin, lactose, and D-xylose.

Although Strain 2 and the proposed neotype culture ATCC 4513 differ in several respects, they both closely resemble *B. circulans* as described in *Bergey's Manual of Determinative Bacteriology* (pages 628 and 629, Seventh Edition, 1957). Variations in the differing characteristics as well as some of the other characteristics are included in this description.

Strains 1 and 2, respectively, have been deposited in the culture collection of Parke, Davis & Co., Detroit, Mich., as P-D 05145 and P-D 05146, and in the ARS Culture Collection of the Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Ill., as NRRL B-3312 and NRRL B-3313.

Fermentation. Early shaken-flask and stirred-jar fermentations, in a medium containing 2.0% glucose monohydrate, 1.0% soybean meal, 0.5% Proto-peptone no. 159 (Wilson & Co.), 0.2% ammonium chloride, 0.5% sodium chloride, and 0.25% calcium carbonate, were extremely erratic. It was subsequently found that glycerol was a better carbohydrate source than glucose and that the type of inoculum, fermentation temperature, and aeration rate were all critical factors in good productivity.

Inoculum for a typical shaken-flask fermentation was prepared in 100 ml of a medium containing 1.0% soybean meal, 1.75% Proto-peptone no. 159 (Wilson & Co.), 0.4% ammonium chloride, and 0.5% calcium carbonate in 1-liter Erlenmeyer flasks and incubated at 30 C for 48 hr on a rotary shaker at 200 rev/min. The same medium supplemented with 4% glycerol was seeded with the 48-hr inoculum and fermented for 5 days under the same conditions used for the inoculum. Typical titers for this fermentation using strain 1 ranged from 100 to 300 μg of butirosin base per ml; with strain 2, titers of 500 to 700 μg of butirosin base per ml were obtained.

A 30-liter, baffled, stirred jar containing 16

liters of the latter medium, which had been adjusted to *p*H 7.5 before addition of calcium carbonate, was inoculated with 5% (v/v) of a 40-hr stirred-jar culture of strain 2 grown on the glycerol-free seed medium. The fermentation was aerated at 0.5 volume of air per volume of medium per min and agitated with a turbine-type impeller at 200 rev/min for 6 days at 30 C. A typical biochemical pattern during such a fermentation is presented in Fig. 1. The first 20 hr were characterized by slight glycerol and ammonia utilization, a decrease in *p*H, and a rapid increase in growth. After 24 hr, the antibiotic titer increased rapidly and at a fairly uniform rate, to a maximum of 630 μg of butirosin base per ml at 116 hr, remained unchanged for the next 20 hr, and decreased to about 550 μg/ml in the final 8 hr. Antibiotic production was paralleled by steady but incomplete glycerol utilization and more rapid and complete ammonia depletion.

The culture grew logarithmically for the first 36 hr of fermentation but remained relatively static during the subsequent period of rapid antibiotic formation. The *p*H decreased rapidly during the active growth phase and then remained fairly stable at about *p*H 6.8 in the biosynthetic phase of fermentation.

Antibacterial activity. Early culture filtrates had shown interestingly diverse antibacterial activity.

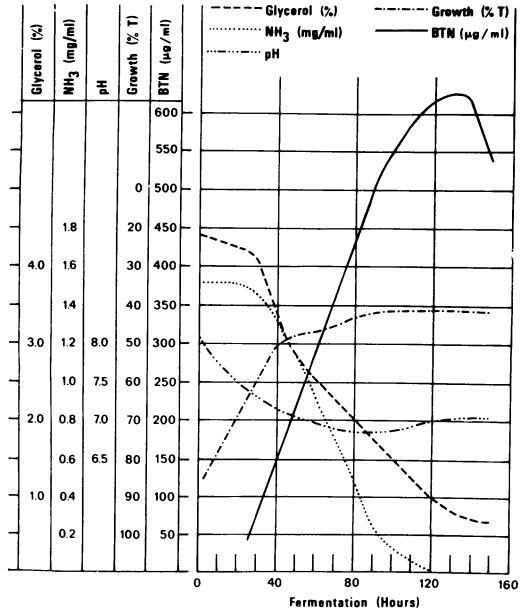

FIG. 1. *Butirosin (BTN) stirred-jar fermentation.*

Filtrates of a fermentation sample that assayed ca. 200 μg of biturosin base per ml showed little or no antifungal activity but gave inhibition zones of 30 mm diameter with *B. subtilis*, 20 mm

TABLE 2. *Antibacterial activity of butirosin (paper-disc, agar-diffusion tests)*

Species	Strain	Zones of inhibition (mm) on butirosin sulfate at (μg/ml)		
		25	6.25	1.56
Aerobacter aerogenes	P-D 0126	21	17 (p)[a]	0
Agrobacterium tumefaciens	P-D 05057	23	19 (p)	0
Bacillus firmus	P-D 05058	23	20 (p)	0
B. metiens	P-D 04161	24	21 (p)	sl
Escherichia coli	P-D 04863	27	24	19
E. coli	Vogel	28	25	21
Klebsiella pneumoniae	P-D 04682	27	23	18
K. pneumoniae	P-D 05076	28	25	20
Proteus mirabilis	P-D 05033	27	21	0
P. vulgaris	P-D 05062	30	24	18
Pseudomonas aeruginosa	P-D 05111	24	18	0
Salmonella schottmuelleri	P-D 01180	28	23	17
S. typhimurium	P-D 05063	25	16	0
S. typhosa	P-D 02481	33	26	20
Shigella dysenteriae	P-D 01339	28	25	21
S. paradysenteriae	P-D 02904	31	27	23
S. sonnei	B 2571	31	28	25
S. sonnei	P-D 04628	27	23	17
Staphylococcus aureus	P-D 02482	29	26	20
S. aureus	P-D 04984	30	25	0
S. aureus	P-D 04988	27	20	0
Streptococcus infrequens	P-D 04664	(sl)[b]	0	0

[a] p, Partial zone.
[b] sl, Slight zone.

TABLE 3. *Antibacterial activity of butirosins A, B, and Complex*

Species and strain	MIC[a] (μg of base/ml of broth)		
	Butirosin A[b]	Butirosin B[b]	Complex[c]
Aerobacter aerogenes, Marshall	3.1	6.3	6.3
Escherichia coli, Vogel	6.3	6.3	6.3
Klebsiella pneumoniae, MGH-1	1.6	3.1	3.1
Proteus mirabilis, MGH-1	12.5	12.5	6.3
P. mirabilis, MGH-3	25	25	25
P. vulgaris, UC-232	6.3	12.5	12.5
P. vulgaris, no. 1810	12.5	6.3	12.5
Pseudomonas aeruginosa, no. 28	3.1	3.1	3.1
P. aeruginosa, 1174C-1	12.5	12.5	6.3
P. aeruginosa, UI-18	3.1	6.3	6.3
P. aeruginosa, VAD S-3	3.1	6.3	12.5
Salmonella typhimurium, V-31	12.5	25	12.5
Shigella sonnei, C-10	6.3	12.5	12.5
Staphylococcus aureus, UC-76	0.80	1.6	0.80
S. aureus, S18713	25	50	25
S. aureus, Bail	3.1	3.1	6.3

[a] Minimal inhibitory concentration, by microtitration of twofold serial dilutions.
[b] Tested as the free base.
[c] Tested as the sulfate, 71% base, containing 80% butirosin A and 20% butirosin B.

with *Staphylococcus aureus*, 24 mm with *E. coli*, 21 mm with *Proteus vulgaris*, 24 mm with *P. aeruginosa*, and 28 mm with *Klebsiella pneumoniae*.

The butirosin sulfate reference standard was also tested against several gram-positive and gram-negative bacteria in a paper-disc, agar-diffusion test employing Mycin Assay Agar (Difco). The results are presented in Table 2. All 20 strains were inhibited by 25 μg of butirosin sulfate per ml and, with the exception of *Streptococcus infrequens*, all were inhibited by 6.25 μg/ml.

Samples of practically pure butirosins A and B base were compared in antibacterial activity and acute toxicity (by our colleague, C. L. Heifetz). In a "microtitration" test adapted from the system of Marymont and Wentz (6) and employing twofold serial dilutions in Tryptic Soy Broth (Difco), no significant difference in antibacterial activity was found between the two bases nor between the complex (reference standard) and the individual bases (Table 3). Acute intravenous toxicity, measured in small groups of mice, also proved to be quite similar for butirosins A and B and the complex with LD_{50} values in the range of 450 to 500 mg/kg for the sulfate salts.

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