BOHOLMYCIN†, A NEW AMINOGLYCOSIDE ANTIBIOTIC

I. PRODUCTION, ISOLATION AND PROPERTIES

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A novel aminoglycoside antibiotic, boholmycin, was produced by *Streptomyces hygroscopicus* H617-25 isolated from a soil sample collected in Bohol Island, the Philippines. It has a pseudotetrasaccharide structure composed of a heptose, two aminosugars and dicarbamoyl-*scyllo*-inositol. Intrinsic antibacterial activity of boholmycin is weak but it exhibits broad spectrum activity against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant strains. Boholmycin is non-toxic in mice at 1,000 mg/kg intravenously.

In the course of our screening program for new antibiotics, a *Streptomyces* strain, No. H617-25, was found to produce a new aminoglycoside antibiotic named boholmycin after the source of its producing organism. The antibiotic showed broad spectrum activity against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant strains, and was effective *in vivo* against bacterial infections. The antibiotic was non-toxic in mice at 1,000 mg/kg, iv. Boholmycin is a new type of aminoglycoside antibiotic structurally unrelated to known antibiotics[1]. This paper describes the taxonomy of the producing organism, production, isolation, physico-chemical and biological properties of boholmycin. The determination of the structure will be the subject of a second publication.

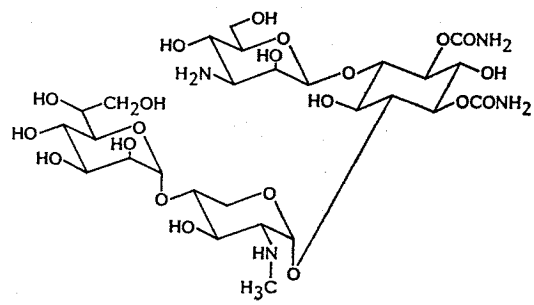
Producing Organism

Strain H617-25 was isolated from a soil sample collected from Bohol Island of the Philippines.

It forms aerial and substrate mycelia with abundant sporulation. The color of the aerial mycelium is white or pale orange yellow, later turns to brownish shade of gray. This strain forms coiled spore-chains on monopodially branched aerial sporophores. Tightly coiled spore-chain containing 10 to 50 arthrospores are often formed. The spores are short-cylindrical, $0.6\sim0.8\times0.8\sim1.2$ μm in size, and have rugose or smooth surfaces. A hygroscopic change on the aerial mycelium occurs often in some agar media such as ISP Nos. 4 and 5.

Strain H617-25 grows well and forms aerial mycelium in both nutritionally rich organic media and chemically defined agar media except for ISP Nos. 3 and 6 media. Strain H617-25 does not produce melanoid pigment in Tryptone - yeast extract broth (ISP No. 1) and peptone - yeast extract - iron agar (ISP No. 6) but does exhibit weak production in tyrosine agar (ISP No. 7). Tyrosinase reaction is negative. Strain H617-25

Fig. 1. The structure of boholmycin.



† Originally called BU-2589 or BMY-28321.

grows on agar medium containing NaCl at 6% but not at 8%. Whorl sporophore, motile spore and sporangium are not observed in any of the media examined. These cultural and physiological characteristics as well as the pattern of carbohydrate utilization of strain H617-25 indicate that it belongs to the genus *Streptomyces*. According to the descriptions in BERGEY's Manual[2], strain H617-25 resembles the species group, *spirales*, gray series, non-chromogenic, and smooth spore surface, which includes 65 species and 7 subspecies. Hygroscopic change of the aerial mycelium (blackening and moistening) is an additional important property of strain H617-25. By comparison of these results with the descriptions in the BERGEY's Manual[2] and those of DIETZ[3], it was concluded that strain H617-25 belongs to the species, *Streptomyces hygroscopicus*.

Antibiotic Production

A well-grown agar slant of *S. hygroscopicus* strain No. H617-25, was used to inoculate a vegetative medium consisting of glycerol 3%, Bacto-liver 1%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.1%, NaCl 0.3% and $CaCO_3$ 0.6%, adjusted to pH 7.0 before sterilization. The medium was incubated at 28°C for 4 days on a rotary shaker (250 rpm). Five ml of the growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the same medium. The fermentation was run at 28°C with shaking on rotary shaker at 250 rpm. The antibiotic activity in the fermentation broth was determined by the paper-disc agar diffusion method using *Bacillus subtilis* PCI 219 as the test organism. A maximal antibiotic potency of approximately 500 μg/ml was obtained after 4 to 5 days fermentation. The fermentation was also carried out in stir-jar fermentors. A 500-ml portion of the seed culture from flask fermentation was inoculated to 12 liters of medium in a 20-liter jar fermentor which was run at 28°C with agitation at 250 rpm and aeration at 10 liters per minute. Antibiotic production reached a maximum of 300 μg/ml after about 90 hours fermentation.

Isolation and Purification

The harvested broth (47 liters, 300 μg/ml) was centrifuged using a Sharpless centrifuge (Kokusan No. 4A). The clarified fermentation liquor was adjusted to pH 7.0 and stirred with Amberlite IRC-50 (6 liters, 60% NH_4^+) to adsorb the antibiotic activity. The resin was washed with water and the antibiotic was eluted batchwise with 0.5 N NH_4OH (10 liters $\times$2). The eluates were pooled and concentrated *in vacuo* to a small volume (300 ml). The concentrate was diluted with an equal volume of methanol and added into acetone (9 liters) to precipitate crude boholmycin (30.1 g). The crude solid was dissolved in 100 ml of water and applied on a column of CM-Sephadex C-25 (2.4 liters, NH_4^+). The column was eluted with a linear gradient of water and 1.0 M NH_4Cl solution (4 liters each). The eluates were collected in 30-ml fractions with an activity peak observed at fraction Nos. 221~240. The combined active fractions were loaded on a column of Sephadex LH-20 (8.4 liters) which was preequilibrated with 50% aqueous methanol. The column was developed with the same solvent and the elution of the antibiotic was monitored by TLC (solvent system, $CHCl_3$ - MeOH - conc NH_4OH - H_2O, 1:4:2:1) and bioassay (*B. subtilis* PCI 219). The fractions containing boholmycin were pooled, concentrated *in vacuo* and lyophilized to afford white powder of boholmycin hydrochloride (13 g).

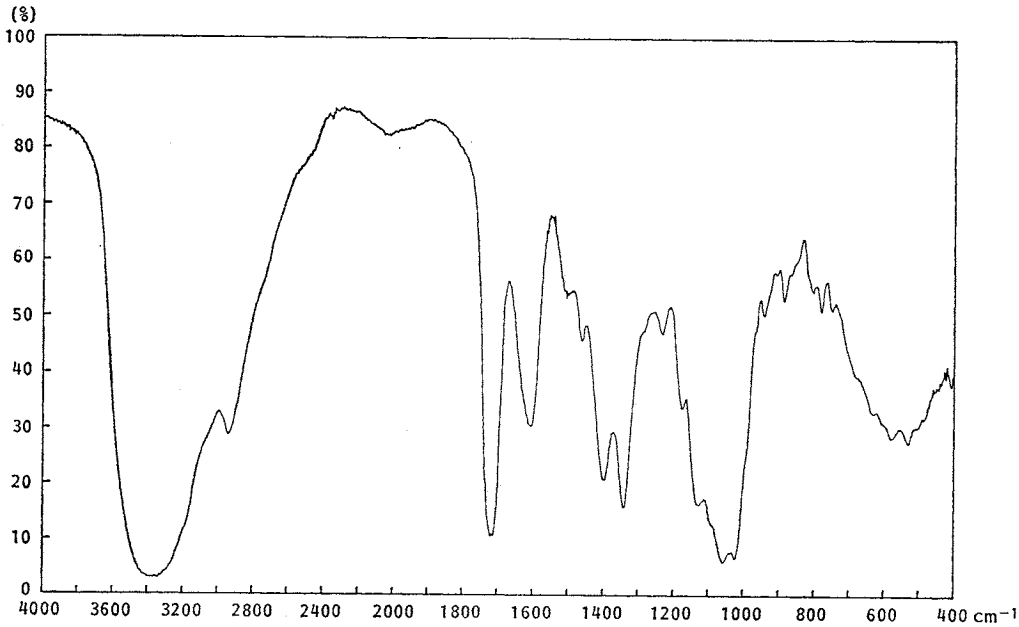
Physico-chemical Properties

Boholmycin is a water-soluble, weakly basic antibiotic. It is freely soluble in water, slightly soluble in methanol and ethanol but practically insoluble in other organic solvents. Boholmycin

Table 1. Physico-chemical properties of boholmycin hydrochloride.

Nature:	White amorphous powder
MP:	214~219°C (dec)
$[\alpha]_D^{28}$ (c 0.5, H_2O):	+52°
Elemental analysis:	
Calcd for $C_{27}H_{48}N_4O_{21}\cdot 2HCl\cdot 4H_2O$:	
	C 35.65, H 6.43, N 6.16, Cl 7.80.
Found:	C 35.44, H 6.00, N 5.96, Cl 8.13.
Mass spectrum (FD-MS):	m/z 787 $(M+Na)^+$, 765 $(M+H)^+$
UV:	No absorption above 210 nm
^{13}C NMR in ppm (multiplicity):	31.7 (q), 56.2 (d), 61.3 (t), 61.4 (d), 61.8 (t), 62.4 (t), 64.1 (d), 68.0 (d), 69.0 (d), 70.6 (d), 71.4 (d), 71.7 (d), 71.9 (d), 72.4 (d), 74.2 (d), 74.4 (d), 76.3 (d), 77.3 (d), 77.7 (d), 81.6 (d), 92.9 (d), 100.9 (d), 102.6 (d), 158.4 (s), 159.2 (s)

Fig. 2. IR spectrum of boholmycin (KBr).



gave a positive reaction in ninhydrin and anthrone tests, but was negative to TOLLEN's and Sakaguchi reagents. The antibiotic was reasonably stable in neutral and acidic solution but unstable in alkaline solution. The physico-chemical properties of boholmycin are summarized in Table 1. It has no UV absorption maximum above 210 nm. The molecular formula was assigned as $C_{27}H_{48}N_4O_{21}$ based on the elemental analysis of its hydrochloride and field desorption mass spectrometry (FD-MS) (M+H m/z 765). The ^{13}C NMR spectrum indicated 25 signals with two of them having a double intensity. The IR spectrum (Fig. 2) shows strong absorption bands at 1720 and 1610 cm^{-1} which are attributable to *O*-carbamoyl group. The ^1H NMR spectrum (Fig. 3) includes three anomeric protons at δ 4.81 (1H, s) 5.02 (1H, d, J=1.8 Hz) and 5.46 (1H, d, J=3.3 Hz), suggesting an oligosaccharide structure for boholmycin. Boholmycin was differentiated by TLC from three aminoglycoside antibiotics,

Fig. 3. ^1H NMR spectrum of boholmycin (400 MHz in D_2O).

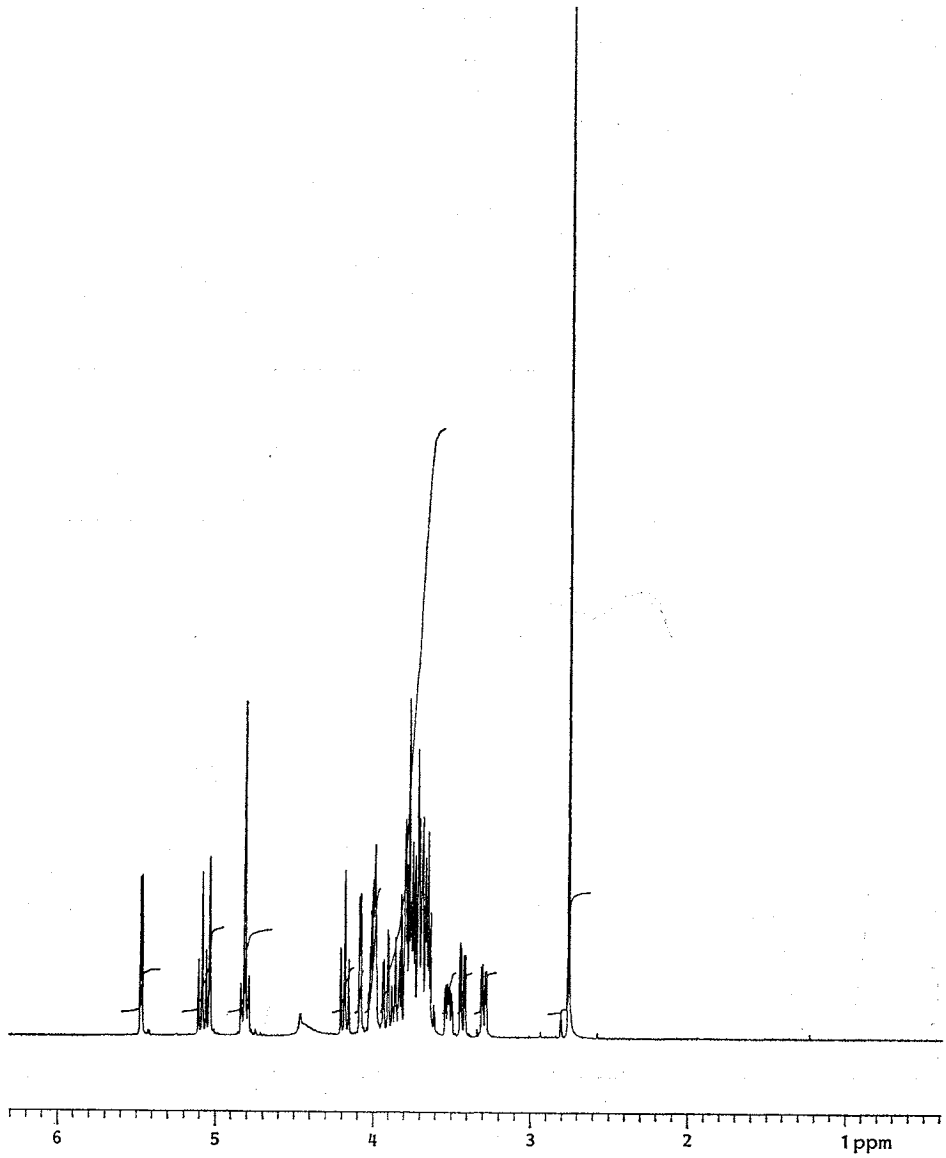


Table 2. TLC comparison of boholmycin and reference antibiotics.

System	TLC Rf value (ninhydrin detection)			
	Boholmycin	Streptomycin	Glebomycin	Myomycin B
S-110	0.28	0.0	0.01	0.0
S-120	0.21	0.15	—	0.04
S-124	0.44	0.41	0.51	—

S-110: SiO_2, $CHCl_3$ - MeOH - conc NH_4OH - H_2O (1 : 4 : 2 : 1), S-120: SiO_2, 95% EtOH - H_2O - AcOH - 2 N NH_4OH (79 : 21 : 5 : 10), S-124: SiO_2, 10% $AcONH_4$ - Me_2CO - conc NH_4OH (9 : 10 : 1).

Table 3. Antibacterial spectra of boholmycin, sorbistin, streptomycin and kanamycin.

Test organisms	Test medium[a]	MIC (μg/ml)			
		Boholmycin	Sorbistin A_1	Streptomycin	Kanamycin
Staphylococcus aureus FDA 209P	A	200	50	1.6	0.8
S. aureus Smith	A	200	50	1.6	0.4
S. aureus A20239[b]	A	>200	100	>100	>100
Micrococcus luteus PCI 1001	A	200	50	3.1	6.3
M. flavus D-12	A	100	12.5	0.8	1.6
Bacillus subtilis ATCC 6633	A	50	50	0.8	0.2
B. anthracis IID-115	A	25	50	0.4	0.4
Escherichia coli NIHJ	A	50	25	1.6	0.8
E. coli Juhl	A	100	50	3.1	1.6
E. coli K-12	A	25	25	1.6	0.8
E. coli A20664[b]	A	12.5	25	>100	>100
E. coli JR35/C600[b]	A	25	25	>100	>100
Klebsiella pneumoniae D-11	A	6.3	6.3	0.4	0.4
K. pneumoniae A9678	A	100	100	6.3	3.1
Enterobacter cloacae A20364[b]	A	50	50	>100	>100
E. cloacae A21006[b]	A	50	50	>100	>100
Proteus mirabilis A9554	A	50	50	1.6	0.8
P. vulgaris A9436	A	6.3	50	0.4	0.2
Serratia marcescens A20019	A	100	>200	25	1.5
S. marcescens A22302[b]	A	100	400	>100	50
Pseudomonas aeruginosa A9930	A	100	12.5	>100	12.5
P. aeruginosa A9843A	A	200	6.3	>100	6.3
P. aeruginosa A20601[b]	A	12.5	3.1	3.1	>100
Mycobacterium smegmatis 607 D87	B	25	50	0.4	0.2
M. smegmatis 607 D46[b]	B	25	100	0.4	>100
M. phlei	B	6.3	25	0.2	0.4
M. ranae	B	25	50	0.4	0.2

[a] A: Nutrient agar (Difco), B: No. 1001 medium (glycerol 3%, sodium L-glutamate 0.3%, peptone 0.2%, $Na_2HPO_4 \cdot 12H_2O$ 0.31%, KH_2PO_4 0.1%, ammonium citrate 0.005%, $MgSO_4$ 0.001%, agar 1.5%).

[b] Kanamycin-resistant strain.

streptomycin, glebomycin[4] and myomycin B[5], which are in some respects chemically related to boholmycin (Table 2).

Biological Properties

In Vitro Antibacterial Activity

The MICs of boholmycin were determined by a serial agar dilution method in comparison with sorbistin A_1[6], streptomycin and kanamycin. Nutrient agar (Difco) was used for all bacteria except for strains of *Mycobacterium* which were tested in No. 1001 agar. As shown in Table 3, the antibacterial activity of boholmycin was comparable to that of sorbistin A_1 but generally weaker than that of streptomycin or kanamycin. However, boholmycin was as active against kanamycin- and streptomycin-resistant organisms as against the sensitive ones. Like the streptomycin-group of antibiotics, boholmycin induced the growth of a streptomycin-dependent strain of *Escherichia coli* D64 (Table 4). Myomycin B was also found to stimulate the growth of this organism.

In Vivo Activity

The *in vivo* efficacy of boholmycin was tested in mice against experimental infections produced by *Staphylococcus aureus*, *E. coli* and *Klebsiella pneumoniae*. Mice were challenged with a multiple

Table 4. Growth induction of streptomycin-dependent *Escherichia coli* D64 by boholmycin and reference antibiotics.

Antibiotic	Conc[a] (mg/ml)	Growth zone[b] of *E. coli* D64 (mm)	Inhibition zone vs.[c] *Bacillus subtilis* PCI-219 (mm)
Boholmycin	40	18 (23)[d]	33
	10	+ (16)	30
Streptomycin	0.25	17 (28)	35
	0.06	+ (14)	32
Glebomycin	4	27	33
	1	15	30
Myomycin B	1	17 (34)	34
	0.25	+ (28)	29
Kanamycin	0.4	—	34
	0.1	—	30

[a] 8 mm paper disc containing 35 μl of test solution.
[b] Incubation at 37°C for 45 hours.
[c] Incubation at 28°C for 18 hours.
[d] ( ) hazy growth zone.

Table 5. *In vivo* activity of boholmycin and reference antibiotics.

Test organisms	PD_{50} (mg/kg, im)			
	Boholmycin	Sorbistin A_1	Streptomycin	Kanamycin
Staphylococcus aureus Smith	75	50	1.9	1.1
Escherichia coli Juhl	100	100	1.4	3.5
Klebsiella pneumoniae D-11	65	75	0.85	1.1

of the lethal dose of the pathogens in a 5%-suspension of gastric mucin (American Laboratory, Omaha, Neb.). Boholmycin was administered intramuscularly just before the bacterial challenge. The mice were observed for 5 days to determine the median protective dose (PD_{50}). Sorbistin A_1, streptomycin and kanamycin were comparatively tested as reference antibiotics. Boholmycin and sorbistin A_1 showed similar *in vivo* activity but were much less active than streptomycin and kanamycin (Table 5). Boholmycin did not show any toxic signs in mice up to a dose of 1,000 mg/kg by intravenous administration.

Discussion

Boholmycin is a novel aminoglycoside antibiotic elaborated by a strain of *S. hygroscopicus* H617-25. Although its intrinsic activity is not potent, boholmycin shows broad antibacterial activity against Gram-positive, Gram-negative and acid-fast bacteria *in vitro* and *in vivo*. Structural studies revealed that boholmycin is a new type of pseudotetrasaccharide antibiotic consisting of a heptose, two amino-sugars and a dicarbamoyl-*scyllo*-inositol (Fig. 1).

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