BALHIMYCIN, A NEW GLYCOPEPTIDE ANTIBIOTIC PRODUCED BY *Amycolatopsis* sp. Y-86,21022

TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITY

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A new glycopeptide antibiotic, balhimycin, has been isolated from the fermentation broth of a *Amycolatopsis* sp. Y-86,21022. Balhimycin belongs to the vancomycin class of glycopeptides and contains a dehydrovancosamine sugar. The biological activity of balhimycin has been compared extensively with that of vancomycin against methicillin resistant staphylococci and also against anaerobes. Balhimycin is marginally superior to vancomycin in its *in vitro* activity against anaerobes and in its bactericidal properties.

In the course of our screening for new antibiotics active against methicillin resistant *Staphylococcus aureus*, a new glycopeptide antibiotic designated as balhimycin (Fig. 1) was isolated from the culture filtrate of an *Amycolatopsis* sp.[1] In this paper we present the taxonomy of the producing organism *Amycolatopsis* sp. Y-86,21022 together with the fermentative production, isolation and biological properties of balhimycin. The unique structural feature of balhimycin is the presence of a dehydrovancosamine sugar moiety and it is one of the first examples of a glycopeptide to contain an α-amino ketosugar.[2),†] The structure elucidation will be presented elsewhere.[3]

Materials and Methods

Microorganism

The strain has been deposited with Deutsche Sammlung von Mikroorganismen (DSM) under the accession number DSM 5908. Chemotaxonomic studies on this culture were carried out at DSM. The cultural characteristics were studied according to the methods of the international *Streptomyces* project (ISP) recommended by SHIRLING and GOTTLIEB[4], WAKSMAN[5] and LECHEVALIER and LECHEVALIER.[6] The utilisation of the carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source at 29°C and observed for two weeks.

Fermentation and Production of Balhimycin

Growth of the organism was evaluated as packed cell volume (PCV) by centrifuging the fermentation

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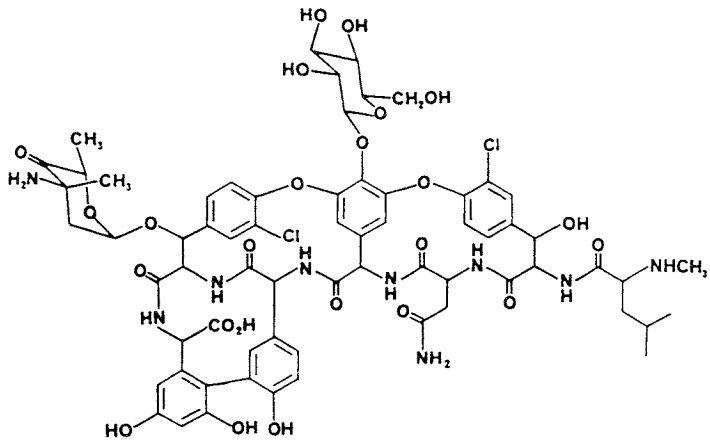
† Subsequent to our results, occurrence of another glycopeptide antibiotic having a dehydrovanosamine unit has been reported [US Patent No. 5187082 dated February 16, 1993. CA, 118:2115430 p, 1993]. The two compounds are structurally different.

Fig. 1. Structure of balhimycin.



broth in a graduated conical tube at $5000 \times g$ for 10 minutes. Glycerol was not analysed.

The content of balhimycin was determined either by the agar-well method using *S. aureus* 3066 as the test organism or by HPLC on a 10 μ ODS-Hypersil [$4 \times (100+30)$ mm] column with a mobile phase of 12% CH_3CN in water containing 0.1% TFA (RT: 7.5 minutes., detection at 280 nm; flow rate: 1 ml/minute).

Determination of Minimum Inhibitory Concentration (MIC)

MIC values were determined by the agar dilution method using Mueller-Hinton (MH) agar for aerobic bacteria and Wilkins-Chalgren (WC) agar for anaerobic bacteria. The aerobic bacteria were precultured in Trypticase-Soy-Broth (TSB) and anaerobic bacteria in thioglycollate broth. About 10^4 cfu/spot (aerobes) or 10^6 cfu/spot (anaerobes) were inoculated with a multipoint replicating apparatus onto agar plates containing serial 2-fold dilutions of the antibiotic. For aerobic bacteria incubation was carried at 37°C for 18 hours. Incubation of anaerobic bacteria was carried out in anaerobic jars (Oxoid) at 37°C for 48 hours. The MIC was read as the lowest drug concentration required to inhibit visible growth of the organism. Growth of less than 5 colonies was considered as negative.

Determination of Minimum Bactericidal Concentration (MBC)

MBC values were determined by the macrodilution broth method in MH broth. The strains precultured in TSB for 18 hours were diluted with fresh TSB to an optical density of 0.3 at 660 nm. Ten μl of this cell suspension was inoculated into 2 ml MH broths containing serially diluted 2-fold dilutions of the drug to give a final inoculum size of 10^6 cfu/ml. After incubation at 37°C for 18 hours, the MIC was read as the lowest drug concentration that inhibited visible growth. 10 Ìl from each clear tube was subcultured onto the surface of a MH agar plate. Plates were incubated at 37°C for 18 hours and the MBC was read as the lowest drug concentration resulting in 99.9% killing of the initial inoculum.

Bactericidal activity was also determined by exposing an initial inoculum of about 10^6 cfu/ml of the test organism to the $4 \times$ MIC concentration of the drug for 6 hours and 24 hours at 37°C in MH broth. Colony counts were performed on these samples using MH agar plates incubated for 18 hours at 37°C. The reduction in cfu/ml from the initial inoculum was expressed as the percent kill.

Post Antibiotics Effect (PAE)

TSB was used as the growth medium in this experiment. Appropriately diluted inoculum obtained from a logarithmically growing broth culture of the test organism was added to drug-free and drug-containing ($4 \times$ MIC) tubes to give an initial inoculum size of 10^6 to 10^7 cfu/ml. The tubes were incubated at 37°C for a period of 1 hour. At the end of 1 hour exposure, 10 μl from the drug-containing and drug-free tubes were added to 9.99 ml of the growth medium prewarmed to 37°C. The diluted samples

were incubated at 37°C and the counts of cfu/ml were performed at time zero *i.e.*, just after dilution and every 1 hour thereafter. The counts, log cfu/ml, were then plotted as a function of time (hours) and the duration of PAE was calculated as the extra time required by the treated sample to increase its cell count by 1 $\log_{10}$ immediately after the drug removal, compared to that required by the untreated sample.

Protein Binding

Plasma protein binding was determined by the ultracentrifugation method described by PETERSON *et al.*[7] It involved ultracentrifugation of drug-plasma mixture (drug concentration: 30 μg/ml plasma) at 295,000 $\times g$ for 3 hours at 24°C and determining the free antibiotic levels in the protein-free supernatant. Protein binding was calculated from the equation

$$\frac{[\text{total drug}]-[\text{free drug}]}{[\text{total drug}]}\times 100 = \%\ \text{bound}\ .$$

Results

Taxonomy of the Producing Strain

No aerial mycelium was observed. The substrate mycelia were well developed, extensively branched and did not fragment into short elements. Conidiospores, sporangia and sclerotia were absent when observed under light microscope.

Data on cultural characteristics and on carbohydrate utilization by strain Y-86,21022 are given in Tables 1 and 2, respectively.

Results of the chemotaxonomic studies carried out at DSM are given in Table 3. Analysis of the

Table 1. Cultural characteristics of strain Y-86,21022.

Medium		Characteristics
Yeast extract-malt extract agar (ISP medium 2)	G:	Good
	AM:	Absent
	RS:	Orange yellow
	SP:	None
Oat meal agar (ISP medium 3)	G:	Poor
	AM:	Absent
	RS:	Light yellow
	SP:	None
Inorganic salts-starch agar (ISP medium 4)	G:	No growth
Glycerol-aspargine agar (ISP medium 5)	G:	Poor
	AM:	Absent
	RS:	Light yellow
	SP:	None
Peptone-yeast extract-iron agar (ISP medium 6)	G:	Poor
	AM:	Absent
	RS:	Whitish
	SP:	None
Tyrosine agar (ISP medium 7)	G:	Good
	AM:	Absent
	RS:	Orange yellow
	SP:	Reddish brown

G, growth; AM, aerial mycelium; RS, reverse side colour; SP, soluble pigment.

Table 2. Utilization of various carbohydrates as the sole source of carbon by strain Y-86,21022.

Carbon source	Utilization	Carbon source	Utilization
D-Glucose	+	Salicin	+
Sucrose	+	L-Arabinose	+
D-Xylose	+	D-Fructose	+
D-Mannitol	+	Dulcitol	−
Inositol	+	Maltose	+
L-Rhamnose	+	D-Galactose	+
Cellulose	−		

+, Growth; −, no growth; ISP medium 9 as the basal medium.

Table 3. Chemotaxonomic characteristics of strain Y-86,21022.

Cell-wall type	IV (*meso*-diaminopimelic acid, arabinose, galactose)
Phospholipid type	PII+OH−PE (DPG, PE, PI, OH-PE)
Menaquinones	MK-9 (H4), (+++); MK-9 (H2), (+)
Mycolic acids	Absent
Cellular fatty acids	*iso*-C_{16}:0, (+++); *iso*-C_{15}:0, (+); *iso*-C_{17}:0, (±); *anteiso*-C_{15}:0 (±); 10 Me-17:0, (+); 2-OH-15:0, (+); 2-OH-16:0, (+)

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; OH-PE, hydroxy-PE; PI, phosphatidylinositol; (±), 1~5%; (+), 5~15%; (++), 15~30%; (+++), >30%.

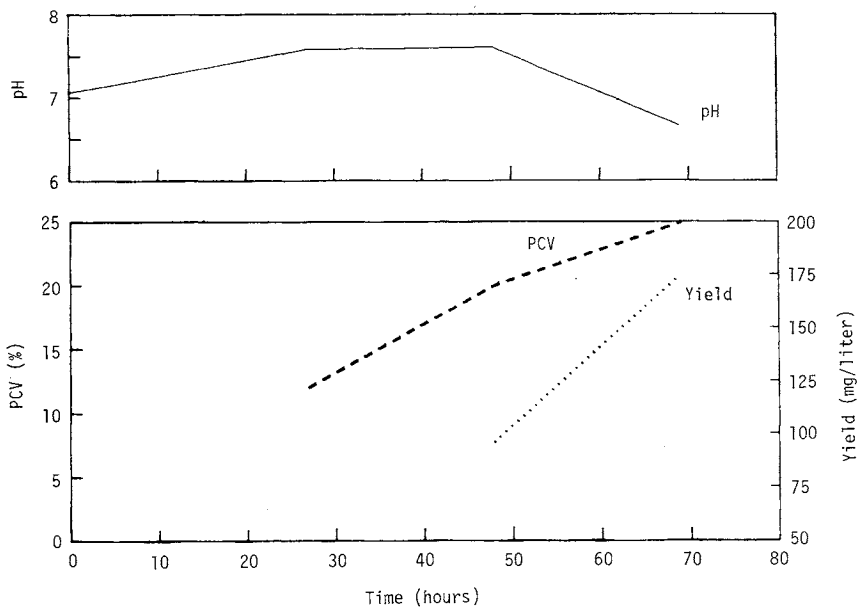
whole cell hydrolysis of strain Y-86,21022 demonstrated the presence of *meso*-diaminopimelic acid, ribose, arabinose and galactose. Accordingly, the cell wall of this strain was classified as type IV of LECHEVALIER and LECHEVALIER.[8] Analysis of the whole-cell phospholipids revealed the presence of diphosphatidylglycerol, phosphatidylethanolamine (PE), hydroxy-PE and phosphatidylinositol (phospholipid type PII+OH−PE). Mycolic acids were not detected. The predominant isoprenoid quinone was MK-9 (H4). Some amount of MK-9 (H2) was also detected. The major component of cellular fatty acids was *iso*-C_{16} : 0.

The taxonomic properties described above and particularly the chemotaxonomic results were in good agreement with those of the genus *Amycolatopsis* but are insufficient to determine the species. *Amycolatopsis orientalis* (DSM strain) used as a control *Amycolatopsis* strain in these studies showed chemotaxonomic properties similar to that of Y-86,21022. Thus the strain Y-86,21022 was identified as an *Amycolatopsis* species.

Fermentation

A seed medium containing glucose 1.5%, soybean meal 1.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% prepared in demineralized water (pH adjusted to 7.0 before autoclaving) was distributed in 160 ml amounts in 1-liter Erlenmeyer flasks and each flask was inoculated with a loopful of cells of strain Y-86,21022 grown for 10 days at 29°C on maintenance medium consisting of malt extract 1%, yeast extract 0.4%, glucose 0.4% and agar 1.3% (pH 7.0). The flasks were shaken on a rotary shaker (240 rpm, 4.0 cm throw) for 72 hours at 29°C. The resultant seed culture (3 liters) was inoculated into 150-liter Marubishi fermentor containing 100 liters of sterile fermentation medium. The medium consisted of glycerol 1.5%, soybean meal 1.0%, $CaCO_3$ 0.1% and NaCl 0.5% (prepared in demineralized water and pH adjusted to 6.8 before autoclaving) along with 30 ml of Desmophen (Bayer) as antifoam. The fermentation was carried out at 29°C for 69~72 hours with aeration (100 liters/minute) and agitation (120 rpm). Under these conditions

Fig. 2. Time course of balhimycin production by *Amycolatopsis* sp. Y-86,21022.



balhimycin was first detected at 48 hours and it reached a maximum concentration at about 72 hours. Balhimycin production measured by HPLC was about 175 mg/liter at 72 hours. The time course of balhimycin fermentation is shown in Fig. 2.

Isolation

The culture filtrate (80 liters, pH 6.5) was passed through Diaion HP-20 (5 liters) and the column was washed with demineralized water (40 liters), 1 M aqueous NaCl (8 liters) and demineralized water (10 liters), respectively. The column was then eluted with 70:30 water-MeOH (9 liters) and finally with 25:75 water-MeOH (51 liters). The eluates were collected in fractions of 1 liter size and the presence of balhimycin was monitored by activity against *S. aureus* 3066. The balhimycin containing fractions, eluted in 25:75 water-MeOH, were combined and concentrated under reduced pressure to remove MeOH and the resulting aqueous solution was passed through Dowex 1×2 OAc^- (1 liter) and washed with demineralized water. The active washings, on concentration and lyophilization, yielded crude balhimycin (21 g) as an off-white powder. It was subjected in two batches of 10 g each to medium pressure liquid chromatography (MPLC) on RP-18 silica gel ($\frac{1}{4}50\sim60$ Ì) packed in a 5.2×54.9 cm Labomatic glass column having a bed volume of 1.23 liters. The column was eluted with 2 liters of water containing 0.1% TFA (v/v) followed by elution with 5% CH_3CN (4 liters), 7.5% CH_3CN (9 liters) and 10% CH_3CN (25 liters). Balhimycin, eluted out in 10% CH_3CN, was collected in fractions of 500 ml and monitored by *in vitro* antibiotic activity as well as by UV detection at 220 nm. The combined active eluates were concentrated under reduced pressure at 40°C and then lyophilized to give 3.15 g of balhimycin as a pale yellow powder. Two such processes gave a total of 6 g of semi-pure balhimycin.

The semi-pure material was finally purified in two batches of 3 g by MPLC on RP-18 using a 3.7×38.2 cm glass column with a bed volume of 410 ml. Elution was carried out using step gradients of CH_3CN in water containing 0.1% TFA in a manner identical to that described above. Balhimycin eluted out in 8 liters of 10% CH_3CN which on concentration and lyophilization gave 900 mg of pure balhimycin trifluoroacetate. From the 6 g of semi-pure balhimycin, 1.8 g of pure balhimycin was obtained.

Balhimycin trifluoroacetate salt was converted to the corresponding acetate salt by passing its solution (1.8 g in 500 ml of water) through Dowex 1×2 OAc^- (450 ml) and washing with water. The active spent and washings were concentrated under reduced pressure and lyophilized to obtained balhimycin acetate salt (1.5 g).

Biological Properties

Biological activity of balhimycin acetate salt was compared against aerobes with that of vancomycin hydrochloride (Eli Lilly). The MICs are shown in Tables 4 and 5. The MIC values of balhimycin are comparable to those of vancomycin against *S. aureus* (methicillin sensitive and resistant), *Staphylococcus epidermidis* (teicoplanin sensitive and resistant) and streptococcal strains. Against teicoplanin resistant strains of *S. epidermidis* and *Staphylococcus haemolyticus*, both balhimycin and vancomycin showed higher MIC values ($2\sim4$ fold higher) than those obtained against teicoplanin sensitive strains. Against Gram-negative bacteria, no activity was observed with either antibiotic.

The MBC/MIC ratios, shown in Table 6, indicate that both balhimycin and vancomycin had good bactericidal activity against different staphylococcal strains but both had poor bactericidal activity against enterococci. A similar trend was also seen in the time-kill experiment (Table 7).

Balhimycin and vancomycin were also tested for their activities against anaerobic bacteria (Table 8).

Table 4. Antibacterial activity of balhimycin and vancomycin.

Test organism	MIC (μg/ml)	
	Balhimycin	Vancomycin
Staphylococcus aureus 209P	0.39	0.78
S. aureus 3066 (Meth^R)	0.78	0.78
S. aureus 20424 (Meth^R)	0.39	0.78
S. epidermidis 178	1.56	1.56
S. epidermidis 825	3.12	1.56
S. epidermidis 823 (Teico^R)	12.50	6.25
S. haemolyticus 712	1.56	1.56
S. haemolyticus 809 (Teico^R)	6.25	3.12
Streptococcus faecalis ATCC 29212	1.56	1.56
S. faecalis D 21777	0.78	1.56
S. faecalis D Endococcen	0.78	0.78
S. faecium D-59	1.56	1.56
S. faecium D-65	0.78	1.56
Escherichia coli 9632	>100.00	>100.00
Proteus rettgeri ATCC 21207	>100.00	>100.00
Pseudomonas aeruginosa NCTC 10701	>100.00	>100.00

Meth^R, Methicillin resistant (Methicillin MIC >100 μg/ml). Teico^R, teicoplanin resistant (Teicoplanin MIC >12.5 μg/ml). MICs were determined by agar dilution method (Point inoculation): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours.

Balhimycin is superior to vancomycin against species of *Peptostreptococcus*, *Propionibacterium* and especially against *Clostridium* species. The activity of both antibiotics against Gram-negative anaerobes is poor.

The post antibiotic effects (PAE) of balhimycin and vancomycin against different test bacteria are

Table 5. Cummulative data on the inhibition of aerobes by balhimycin and vancomycin.

Test organism (n=number of strains)	MIC_{90} (μg/ml)	
	Balhimycin	Vancomycin
Staphylococcus aureus (Meth^S) (n=120)	0.5	0.5
S. aureus (Meth^R) (n=39)	1.0	1.0
S. epidermidis (n=20)	2.0	1.0
Streptococcus faecalis (n=40)	1.0	2.0
S. faecium (n=36)	1.0	1.0

Meth^S, Methicillin sensitive (MIC <6.25 μg/ml) *S. aureus*. Meth^R, methicillin resistant (MIC >12.5~200 μg/ml) *S. aureus*. Agar dilution method (Point inoculation): Mueller-Hinton agar, 10^4 cfu/spot, 37°C, 18 hours.

Table 6. MBC/MIC values of balhimycin and vancomycin.

Test organism	MIC (μg/ml)		MBC/MIC	
	Balhimycin	Vancomycin	Balhimycin	Vancomycin
Staphylococcus aureus 3066 (Meth^R)	0.39	0.39	2	2
S. aureus 20424 (Meth^R)	0.39	0.39	2	1
S. epidermidis 825	1.56	0.78	2	2
S. haemolyticus 712	1.56	0.78	2	4
Streptococcus faecalis ATCC 29212	0.78	0.78	16	32
S. faecalis D 21777	0.78	1.56	64	32
S. faecium D-59	1.56	3.12	32	16

Macrodilution method: Mueller-Hinton broth, 10^6 cfu/ml, 37°C, 18 hours. MBC: killing 99.9% of the inoculum.

Table 7. Bactericidal activity of balhimycin and vancomycin (10^6 cfu/ml).

Test organism (MIC: μg/ml) (Balhimycin) (Vancomycin)	% kill at 4 MIC			
	6 hours		24 hours	
	Balhimycin	Vancomycin	Balhimycin	Vancomycin
Staphylococcus aureus 3066 (0.78) (0.78)	99.9	98.7	99.9	99.9
S. epidermidis 178 (1.56) (1.56)	99.7	98.4	99.9	99.9
Streptococcus faecalis D 21777 (0.78) (1.56)	87.4	80.0	98.7	99.0

Table 8. Antibacterial activity of balhimycin and vancomycin against anaerobes.

Test organism	MIC (μg/ml)	
	Balhimycin	Vancomycin
Peptostreptococcus anaerobius 932	0.39	0.78
Propionibacterium acnes 6919	0.19	0.78
P. acnes 6922	0.19	0.78
Clostridium tetani 19406	0.39	12.50
C. perfringens 194	0.10	0.78
Bacteroides fragilis 960	25.00	50.00
Fusobacterium varium 5262	>100.00	>100.00

Agar dilution method (Point inoculation); Wilkins Chalgren Agar; Inoculum: 10^6 cfu/spot; Incubation: 37°C; 48 hours in anaerobic jars.

shown in Table 9. Balhimycin showed better PAE compared to vancomycin, although marginally.

Plasma protein binding results are given in Table 10. Binding for balhimycin (35~40%) is marginally higher than that for vancomycin (30~35%).

Table 9. Post-antibiotic effect (PAE) of balhimycin and vancomycin.

Test organism	PAE (hours)	
	Balhimycin	Vancomycin
Staphylococcus aureus 3066	4	2
S. aureus E710	4	3
S. epidermidis 32965	5	4
S. epidermidis 9879	1.25	1.0
Streptococcus faecalis D 21777	1.25	0.5
S. faecium 29212	0.5	nil

Bacteria were exposed to the drugs for 1 hour. Drug concentration: 4×MIC.

Table 10. Human plasma protein binding of balhimycin and vancomycin.

Antibiotic	Plasma protein binding (%)	
	Experiment 1	Experiment 2
Balhimycin	35	30
Vancomycin	40	35

Ultracentrifugation method; drug concentration: 30 μg/ml.

Discussion

A new glycopeptide antibiotic, designated balhimycin, was isolated from the fermentation broth of an *Amycolatopsis* sp. Balhimycin and vancomycin showed equal activity against aerobes including methicillin resistant *S. aureus* strains. Against anaerobes activity of balhimycin was found to be superior to vancomycin particularly against *Clostridium* strains. It also showed better bactericidal activity and post-antibiotic effect compared to vancomycin. The interesting feature of balhimycin is the presence of dehydrovancosamine in its chemical structure which makes it readily amenable to structural modifications in this part of the molecule. Its toxicity, chemotherapeutic effectiveness are comparable to that of vancomycin and will form part of a separate presentation.

Acknowledgments

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