GLYSPERIN, A NEW ANTIBIOTIC COMPLEX OF BACTERIAL ORIGIN

I. PRODUCTION, ISOLATION AND PROPERTIES

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Strains of *Bacillus cereus* produced a complex of new antibiotics, glysperins A, B and C. They are basic, water-soluble antibiotics and active against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant organisms. Glysperin A is a major component of the antibiotic complex and approximately two to four times more active than components B and C.

In the course of our screening for new antibiotics produced by microorganisms of the order *Eubacteriales,* two strains of *Bacillus cereus,* Nos. F173-B61 and F262-B54, were found to produce a basic, water-soluble antibiotic complex, glysperin*, having a broad antibacterial spectrum. The antibiotic complex in fermentation broth was isolated by using a cation exchange resin and then separated into three components A, B and C by chromatographic procedures. As described in a companion paper[1], glysperins A, B and C possess a new type of chemical structure consisting of a diaminohexose-containing tetrasaccharide and a *p*-hydroxybenzoyl polyamine. This paper reports on the production, isolation, characterization and biological properties of glysperin.

Producing Organism

Two strains of the glysperin-producing organism, F173-B61 and F262-B54, were isolated from soil samples collected in West Germany and India, respectively. Both strains are aerobic, catalase-positive, Gram-positive, spore-forming rods and thus they belong to the genus *Bacillus.* The morphological characteristics of strains F173-B61 and F262-B54 resemble those of *Bacillus cereus* or *Bacillus megaterium* in that they are Gram-positive motile rods of similar size (1.0 ~ 1.2 by 1.5 ~ 4.5 μm), bear oval spores at the central position without distension and form intracellular globules unstainable by fuchsin. The cultural and physiological characteristics of strains F173-B61 and F262-B54 are summarized in Table 1 along with those of *B. cereus* and *B. megaterium.* Strain F173-B61 differs from strain F262-B54 in its growth under anaerobic conditions, its positive egg-yolk reaction, nitrate reduction and VP-reaction. The physiological characteristics of strains F173-B61 and F262-B54 resemble those of *Bacillus cereus/megaterium* intermediate strains described by KNIGHT and PROOM[3]. However, F173-B61 and F262-B54 are resistant to the lytic action of lysozyme, which is like *B. cereus* but unlike *B. megaterium.* For further taxonomical identification, the bacterial DNA's of the two strains were extracted according to the method of SIGAL *et al.*[4] and the DNA base compositions analyzed by the chemical method of BENDICH[5]. The guanine-plus-cytosine (GC) content of strains F173-B61 and F262-B54 was determined to be 34.1 ± 0.5% and 34.8 ± 0.6%, respectively, which are in the range of

* This antibiotic was originally designated as Bu-2349.

Table 1. Cultural and physiological characteristics.

	Strain F173-B61	Strain F262-B54	*Bacillus cereus* ATCC 10702	*Bacillus megaterium* ATCC 14945
Cell mass growth in glucose nitrate broth and tryptosoy broth	Floccose, sedimented and white; not viscous	Floccose, sedimented and white; not viscous	Floccose, sedimented and white; not viscous	Floccose, sedimented and white; not viscous
Colony on nutrient agar (28°C, 6 days)				
Color	Pale yellow	Pale yellow	Pale yellow	Pale yellow
Extreme	Heaped, non-spreading	Heaped, non-spreading	Diffused, root-like outgrowth	Heaped, non-spreading
Surface	Slightly rugose, pustular	Slightly rugose, pustular	Dull, frosted glass appearance	Slightly rugose, pustular
Size (mm in diameter)	10~12	8~10	18~24	6~8
Growth-temperature:				
Abundant growth	20~45°C	20~45°C	20~45°C	10~40°C
No growth	10°C, 50°C	10°C, 50°C	10°C, 50°C	5°C, 45°C
Acid in glucose broth	+	+	+	+
Gas from glucose	−	−	−	−
Acid from arabinose, xylose and mannitol	−	−	−	−
Anaerobic growth in HUGH & LEIFSON medium	+	−	+	−
Growth in 0.001 %-lysozyme	+	+	+	−
Lysis by lysozyme (%)[2]	0	0	0	87
Nitrite from nitrate	+	−	+	+
Egg-yolk reaction	+	−	+	−
Acetoin from glucose	+	variable	+	−
Gelatin liquefaction	+	+	+	+
Starch hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Alkali on citrate salts agar	+	+	+	+
Catalase	+	+	+	+
Growth at 7 % sodium chloride	+	+	+	+
Growth in ammonium-salts medium	+	+	−	+
Requirement of vitamine or amino acid for growth	−	−	+	−

that reported for *B. cereus* (33.3~36.0%) rather than that of *B. megaterium* (36.0~37.6%)[6]. Thus, the two strains were concluded to belong to the species, *B. cereus*. They have been deposited in the American Type Culture Collection and assigned the designation ATCC 31429 for strain F173-B61 and ATCC 31430 for strain F262-B54.

Antibiotic Production

Strain F173-B61 was discovered first to elaborate the new antibiotic glysperin. A second producing organism, strain F262-B54, was subsequently isolated and used for further study because of its higher

productivity. A well-grown agar slant of F262-B54 was inoculated into vegetative medium containing 1% glucose, 0.5% yeast extract and 1% polypeptone. The pH of the medium was adjusted to 7.2 before sterilization. The seed culture was incubated at 28°C for 24 hours on a rotary shaker (250 rpm), and 5-ml portions of the growth were transferred to 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium composed of 3% glycerol, 0.5% soybean meal, 1% fish meal, 0.1% $(NH_4)_2SO_4$, 0.3% NaCl and 0.6% $CaCO_3$. Antibiotic production reached a maximum after 4~6 days shaking at 28°C. The antibiotic activity in the fermentation broth was determined by a paper disc - agar diffusion method using *Bacillus subtilis* PCI 219 as the test organism.

Fermentation studies were also performed in 20-liter jar fermentors containing 10 liters of medium which consisted of 4.5% glycerol, 0.8% soybean meal, 1.5% fish meal, 0.1% $(NH_4)_2SO_4$, 0.3% NaCl and 0.6% $CaCO_3$. Fermentation temperature was adjusted to 28°C for the first 20 hours and to 32°C thereafter. A peak antibiotic potency of 150~200 mcg/ml was obtained after 90~95 hours' fermentation.

Isolation and Purification

The harvested broth was adjusted to pH 6.5 with an addition of oxalic acid, stirred for 30 minutes and then filtered with filter aid. The antibiotic activity in the filtrate was adsorbed on a column of Amberlite IRC-50 (NH_4^+ form). The column was washed with water and 0.1 N NH_4OH successively, and then developed with 2 N NH_4OH. Active fractions were pooled and concentrated *in vacuo* to afford the glysperin complex as a crude solid.

This was chromatographed using a column of Amberlite CG-50 and the column was developed with increasing concentrations of aqueous ammonia. Glysperin B was eluted first with 0.5 N NH_4OH and a mixture of glysperins A and C with 1 N NH_4OH. The mixture of A and C components was dissolved in water, adjusted to pH 7.0 with dil.HCl and charged on a column of Diaion HP-20 AG, the column being pre-equilibrated with 0.05 M phosphate buffer (pH 7.0). Development of the column with water afforded glysperin C first, followed by glysperin A. Overlapping fractions were rechromatographed on the HP-20 column to achieve complete separation of components A and C. Each single component was further purified by Amberlite CG-50 chromatography for characterization. An example of the relative yield of glysperin components was A (4.23 g), B (530 mg) and C (610 mg) from 45 liters of fermentation broth.

Physico-chemical Properties

Glysperins A, B and C were obtained as white amorphous carbonates after the above-described extraction and purification procedure. Two TLC solvent systems, S-102 and S-114, were suitable for differentiating glysperin B from glysperins A and C (Table 2). The latter two components were sepa-

Table 2. TLC of glysperin components.

	Solvent system	Rf		
		A	B	C
S-102	MeOH - 10%$AcONH_4$ (1 : 1)	0.14	0.31	0.13
S-114	MeOAc - *n*-PrOH - 28%NH_4OH (45 : 105 : 60)	0.06	0.16	0.06
S-117	$CHCl_3$ - MeOH - 28%NH_4OH (1 : 3 : 2)	0.36	0.50	0.30

rated from each other by solvent system S-117. The three glysperin components are readily soluble in water, slightly soluble in methanol, ethanol, dimethylformamide and dimethylsulfoxide, and practically insoluble in other organic solvents. They showed positive reactions with ninhydrin, anthrone, RIMINI[7] and ELSON-MORGAN reagents but were negative to ferric chloride, TOLLENS and SAKAGUCHI tests.

Physico-chemical properties of glysperins A, B and C are summarized in Table 3. They exhibited

Table 3. Physico-chemical properties of glysperins A, B and C.

	Glysperin A		Glysperin B		Glysperin C	
Nature	Basic colorless powder		Basic colorless powder		Basic colorless powder	
M.p.	132~137°C (dec.)		166°C (dec.)		140~145°C (dec.)	
$[\alpha]_D^{25}$ (c 0.5, H_2O)	+113°		+132°		+157°	
UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	247 (126)		247 (147)		247 (133)	
Molecular formula	$C_{44}H_{75}N_7O_{18}$		$C_{40}H_{66}N_6O_{18}$		$C_{44}H_{77}N_7O_{19}$	
Elemental analysis	$C_{44}H_{75}N_7O_{18}\cdot 2H_2CO_3$		$C_{40}H_{66}N_6O_{18}\cdot 3/2\,H_2CO_3$		$C_{44}H_{77}N_7O_{19}\cdot 2H_2CO_3$	
	Calc'd	Found	Calc'd	Found	Calc'd	Found
C %	49.59	49.30	49.25	49.25	48.80	48.79
H %	7.15	7.32	6.87	7.19	7.21	7.41
N %	8.80	8.47	8.30	8.31	8.66	8.81
NMR spectrum ($\delta_{DSS}^{D_2O}$ ppm) Characteristic signals	1.28 (d, J=6.5 Hz, 3H)		1.26 (d, J=6.5 Hz, 3H)		1.27 (d, J=6.5 Hz, 3H)	
	1.49 (d, J=7.0 Hz, 3H)		1.46 (d, J=7.0 Hz, 3H)		1.52 (d, J=7.0 Hz, 3H)	
	1.6~2.4 (m, 10H)		1.6~2.4 (m, 6H)		1.6~2.4 (m, 10)	
	5.23 (d, J=2 Hz, 1H)		5.21 (d, J=2 Hz, 1H)			
	5.84 (d, J=4.0 Hz, 1H)		5.82 (d, J=4.0 Hz, 1H)		5.85 (d, J=4.0 Hz, 1H)	
	7.12 (d, J=9.0 Hz, 2H)		7.09 (d, J=9.0 Hz, 2H)		7.17 (d, J=9.0 Hz, 2H)	
	7.71 (d, J=9.0 Hz, 2H)		7.69 (d, J=9.0 Hz, 2H)		7.77 (d, J=9.0 Hz, 2H)	

Fig. 1. IR Spectrum of glysperin A hydrochloride.

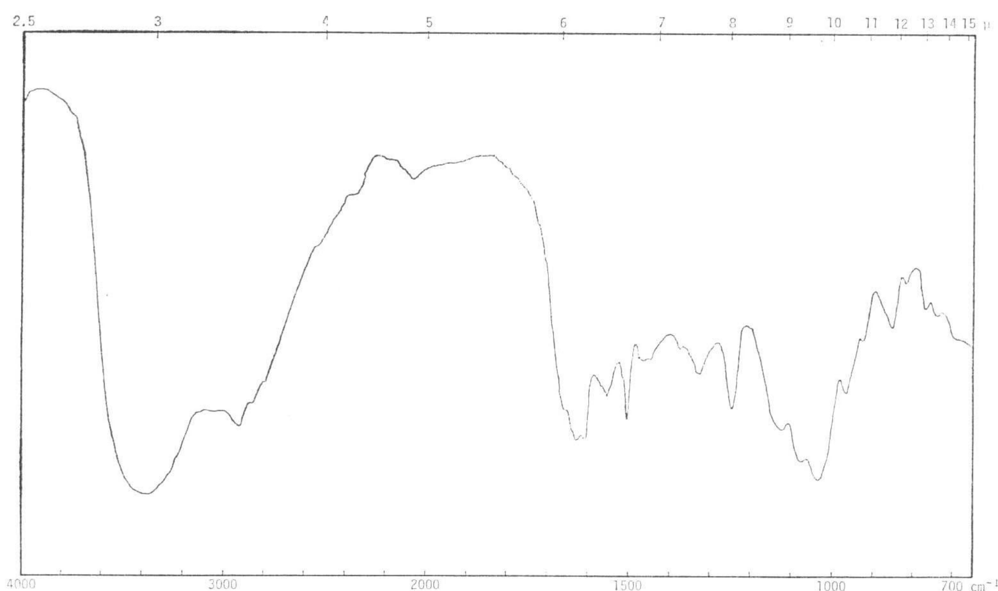


Fig. 2. NMR Spectrum of glysperin A hydrochloride.

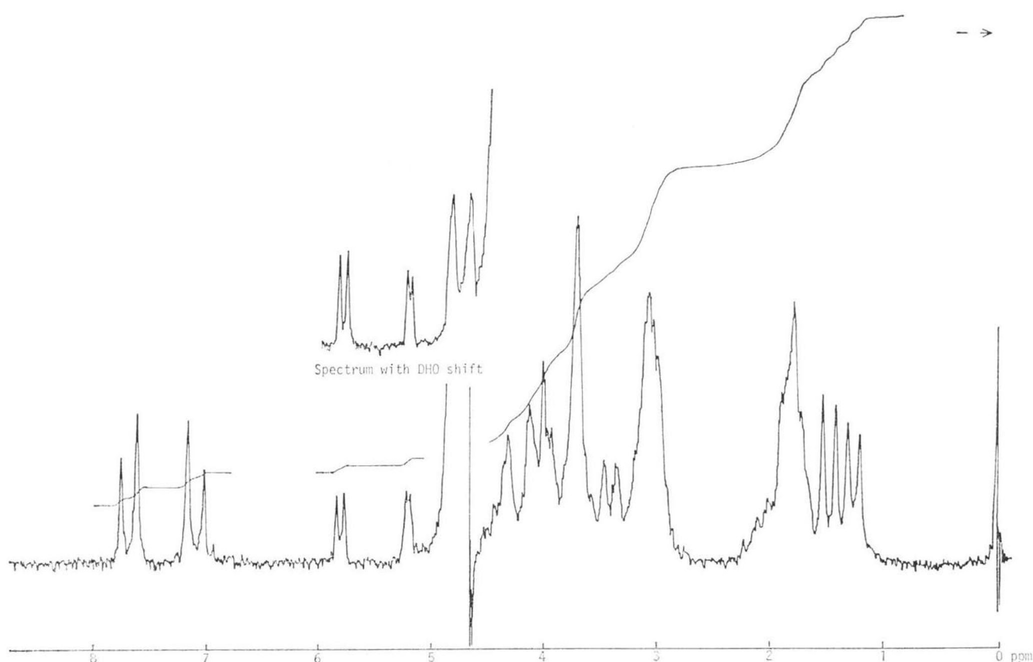
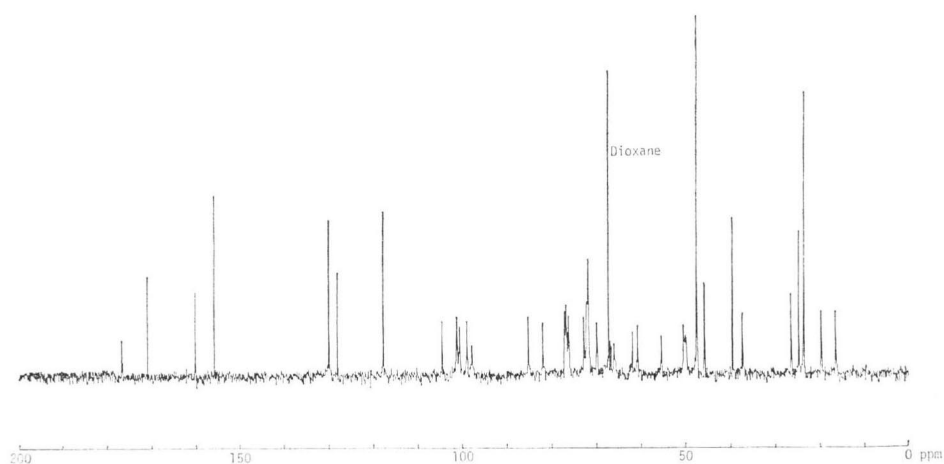


Fig. 3. ^{13}C-NMR Spectrum of glysperin A.



a single UV absorption maximum at 247 nm in water and no shift was observed in acidic or alkaline solution. The IR spectrum of glysperin A (Fig. 1) was nearly identical with that of the B and C components, showing amide carbonyl bands at 1635 and 1560 cm^{-1} and polyhydroxyl absorptions at around 3400 and 1040 cm^{-1}. The NMR spectrum of glysperin A hydrochloride (Fig. 2) is similar to that of glysperins B and C. It includes signals for two methyl groups, four aromatic protons and several protons in the anomeric region. The CMR spectrum of glysperin A (Fig. 3) indicated the presence of a total of 44 carbons.

Antimicrobial Activity

The minimum inhibitory concentrations (MIC) of glysperins A, B and C were determined by means of a two-fold serial dilution method in MUELLER-HINTON agar medium (Difco, Detroit) against standard laboratory strains of Gram-positive, Gram-negative and acid-fast bacteria. Surfaces of the agar plates were inoculated with approximately 10^4 CFU (colony forming units) of test organisms using a Steers multi-inoculating apparatus. The MIC was defined as the lowest concentration of test compound completely inhibiting bacterial growth after overnight incubation at 37°C. The *in vitro* antibacterial spectra of glysperins A, B and C are shown in Table 4 along with that of kanamycin A which was tested comparatively as a reference compound. The glysperin antibiotics were active against Gram-positive, Gram-negative and acid-fast bacteria. The intrinsic activity of glysperin A was generally 2~4 fold greater than that of glysperins B and C but was about one-half that of kanamycin A when tested in MUELLER-HINTON agar medium.

The MIC of glysperin A was also determined by a two-fold tube-dilution method using two kinds of liquid media, MUELLER-HINTON broth (Difco) and nutrient broth (Difco). The inoculum size was adjusted to $10^4 \sim 10^5$ CFU of each test organism per ml of the liquid medium (pH 7.0). The results are shown in Table 5 compared with kanamycin A. The *in vitro* activity of glysperin A determined in liquid media, especially in nutrient broth, was significantly higher than that measured in MUELLER-HINTON agar medium. In contrast to the activity ratio obtained by the agar-dilution method, glysperin A was more active against most Gram-negative bacteria in the tube dilution method than kanamycin A.

Various types of aminoglycoside-resistant organisms which have been shown to produce aminoglycoside-modifying enzymes were examined in MUELLER-HINTON agar for their sensitivity toward gly-

Table 4. Antibacterial spectra of glysperins A, B and C (Agar-dilution method in MUELLER-HINTON agar).

Test organisms	MIC (mcg/ml)			
	Glysperin			Kanamycin A
	A	B	C	
Staphylococcus aureus FDA 209P	1.6	6.3	3.1	0.8
〃 Smith	1.6	6.3	3.1	0.8
Bacillus subtilis PCI 219	1.6	25	6.3	0.4
Bacillus brevis ATCC 8185	0.8	1.6	3.1	0.8
Streptococcus pneumoniae A9585	>100	>100	>100	25
Escherichia coli NIHJ	1.6	3.1	3.1	0.8
〃 Juhl	3.1	6.3	12.5	3.1
〃 ML-1630	1.6	3.1	6.3	>100
Klebsiella pneumoniae D11	1.6	6.3	6.3	0.8
Enterobacter cloacae A9656	3.1	12.5	12.5	3.1
Proteus mirabilis A9900	12.5	25	12.5	1.6
Proteus vulgaris A9699	3.1	25	6.3	1.6
Serratia marcescens A20019	50	50	50	3.1
Pseudomonas aeruginosa A9843	>100	>100	>100	>100
Mycobacterium smegmatis 607	1.6	3.1	1.6	0.4
Mycobacterium phlei D88	1.6	3.1	1.6	0.4
Mycobacterium ranae ATCC 110	1.6	3.1	1.6	0.4

sperin A and reference aminoglycoside antibiotics. As shown in Table 6, these resistant organisms were fully susceptible to glysperin A. The *in vitro* activity of glysperin A was also compared with that of kanamycin, gentamicin and cefazolin against clinical isolates of Gram-positive and Gram-negative bacteria. The results are summarized in Table 7. Glysperin A showed a relatively small range of

Table 5. Antibacterial activity of glysperin A (Broth dilution method).

Test organisms	MIC (mcg/ml)			
	MUELLER-HINTON broth		Nutrient broth	
	Glysperin A	Kanamycin A	Glysperin A	Kanamycin A
Staphylococcus aureus FDA 209P	0.4	0.2	0.05	0.05
〃 Smith	0.8	0.4	0.1	0.025
Escherichia coli NIHJ	0.2	1.6	0.05	0.2
〃 Juhl	0.8	3.1	0.05	0.2
Klebsiella pneumoniae D11	0.1	0.2	0.002	0.008
Proteus mirabilis A9900	1.6	3.1	0.025	0.8
Proteus vulgaris A9699	0.4	1.6	0.013	0.4
Serratia marcescens A20019	6.3	1.6	0.2	0.4
Pseudomonas aeruginosa A9843	100	25	12.5	1.6

Table 6. Activity of glysperin A against aminoglycoside-resistant organisms (MUELLER-HINTON agar).

Test organisms	Enzymes*	MIC (mcg/ml)				
		Glysperin A	Kanamycin A	Gentamicin C	Tobramycin	Amikacin
Escherichia coli NR79/W677	AAC (6′)-I	0.8	>100	0.4	0.4	0.8
〃 JR/C600	APH (3′)-I	1.6	>100	0.4	0.4	0.4
〃 A20107	APH (3′)-II	1.6	>100	0.8	0.8	1.6
〃 JR88	AAC (3)-I	1.6	1.6	25	0.4	0.8
Klebsiella pneumoniae 22-3038	APH (3′)-II & ANT (2″)	6.3	>100	25	12.5	3.1
Providencia stuartii A20894	AAC (2′)	3.1	1.6	6.3	6.3	1.6
Staphylococcus aureus A20239	APH (3′)-I, II	3.1	>100	0.4	0.2	1.6
〃 A21978	ANT (4′)	6.3	>100	1.6	>100	25

* Abbreviation for aminoglycoside-modifying enzymes: see ref. 8.

Table 7. *In vitro* susceptibility of clinical isolates (MUELLER-HINTON agar).

Test organisms	No. strains	Range of MIC (mcg/ml)			
		Glysperin A	Kanamycin A	Gentamicin C	Cefazolin
Staphylococcus aureus	10	0.4∼ 12.5	0.2∼ 6.3	0.05∼ 0.8	0.2∼ 1.6
Escherichia coli	5	1.6∼ 6.3	1.6∼ 6.3	0.8 ∼ 3.1	1.6∼>100
Klebsiella pneumoniae	4	1.6∼ 12.5	0.8∼>100	0.4 ∼50	3.1∼>100
Enterobacter cloacae	7	3.1∼ 12.5	3.1∼>100	0.8 ∼ 1.6	>100
Proteus morganii	5	12.5∼ 50	1.6∼ 6.3	0.8 ∼ 1.6	>100
Serratia marcescens	10	25 ∼>100	3.1∼>100	0.8 ∼50	>100
Pseudomonas aeruginosa	6	100 ∼>100	25 ∼>100	0.8 ∼ 6.3	>100

MICs for varied species of bacterial isolates, except for strains of *P. aeruginosa* and *S. marcescens* which were generally resistant to the antibiotic.

Strains of *S. marcescens* are known to produce spermidine oxidase[9] which cleaves spermidine into 1,3-diaminopropane and γ-aminobutyraldehyde (then to Δ^1-pyrroline). As reported in a companion paper[1], glysperins A, B and C have a spermidine or a spermidine-like polyamine moiety in the terminal side-chain of their molecular structure, and hence it was suspected that there could be a correlation between the production of spermidine oxidase and the resistance to glysperin in strains of *S. marcescens*. Among 20 cultures of *S. marcescens* examined for spermidine oxidase and its relationship to antibiotic resistance, 15 resistant strains (MIC of glysperin A: $\geqq$50 mcg/ml) were found to be high producers of the enzyme.

The *in vivo* efficacy of glysperin A was assessed by experimental systemic infections in mice. The pathogenic bacteria used in the *in vivo* tests were *S. aureus* Smith, *E. coli* Juhl, *K. pneumoniae* A9977, *P. mirabilis* A9554 and *P. vulgaris* A9436. Mice were challenged intraperitoneally with a *ca.* 100 LD_{50} dose of the pathogens in a 5% suspension of hog gastric mucin (American Laboratories, Omaha). Antibiotics were administered intramuscularly immediately after the bacterial challenge. A group of 5 mice was used for each dose level and the animals were observed daily for 5 days to determine the median protective dose (PD_{50}). The results are compared in Table 8 with those of kanamycin A. Glysperin A was effective at relatively low doses against Gram-positive and Gram-negative infections. The median lethal doses (LD_{50}) of glysperin A determined in mice by intramuscular and intravenous routes were 285 mg/kg and 35 mg/kg, respectively.

Table 8. *In vivo* activity of glysperin. A.

Test organisms	PD_{50} (mg/kg, i.m.)	
	Glysperin A	Kanamycin A
Staphylococcus aureus Smith	2.5	1.1
Escherichia coli Juhl	4.0	5.0
Klebsiella pneumoniae A9977	3.8	3.3
Proteus mirabilis A9554	16	1.6
Proteus vulgaris A9436	16	1.8

Discussion

Glysperin is a complex of basic, water-soluble antibiotics produced by strains of *B. cereus*. In recent years there have been several examples of the production of basic, water-soluble antibiotics by bacterial strains, such as butirosins A and B[10], Bu-1709 E_1 and E_2[11], xylostasin[12], Bu-1975 C_1 and C_2[13], and sorbistin[14]. Except for sorbistin, they are all aminocyclitol-containing aminoglycoside antibiotics produced by species of *Bacillus*. Although glysperin contains an aminosugar moiety in its structure[1], it is not a classical aminoglycoside antibiotic and hence most aminoglycoside-modifying enzymes now known have no effect on its antimicrobial activity. Strains of *S. marcescens* which produced spermidine oxidase were resistant to the antibiotic.

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