EM49, A NEW PEPTIDE ANTIBIOTIC

III. BIOLOGICAL CHARACTERIZATION *IN VITRO* AND *IN VIVO*

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EM49, a novel peptide antibiotic produced by *Bacillus circulans*, shows broad-spectrum antibacterial activity *in vitro* and also has substantial activity against yeasts, fungi, and protozoa. Its high degree of antipseudomonal activity and its greater activity against gram-negative than against gram-positive bacteria are noteworthy. EM49 is rapidly biocidal to populations of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Even after ten successive subcultures in the presence of EM49, strains of *P. aeruginosa*, *E. coli* and *S. aureus* did not develop any resistance to this compound. The antibiotic was active *in vivo* upon subcutaneous administration to mice infected with *Streptococcus pyogenes* C203 or *E. coli*, but was inactive when given by the oral route. When applied topically at a concentration of 0.5% in a cream base, EM49 prevented the multiplication of *P. aeruginosa* in experimentally induced wounds in mice.

The discovery of a new, broad-spectrum, peptide antibiotic produced by *Bacillus circulans* ATCC 21,656 has been reported[1]. The antibiotic is a mixture of octapeptides, presumably cyclic, that are acylated with a C_{10} or C_{11} fatty acid side chain[2]. Though closely related to the polymyxin group of antibiotics, EM49 is distinguished from the polymyxins by a difference in the number of amino acid residues, by a lack of threonine, and by differences in the nature of the fatty acid moieties with respect to the number of carbon atoms and the presence of a β-hydroxy group[2]. The lack of cross-resistance between EM49 and polymyxin B, and the greater antistaphylococcal activity of EM49, also serve to differentiate these compounds[1]. In this manuscript, we describe our studies on the behavior of this antibiotic *in vitro* and *in vivo* and on the taxonomic characterization of the producing organism, *Bacillus circulans* ATCC 21,656.

Experimental

All minimal inhibitory concentration values (MIC) described in this report were determined by a standard, twofold broth-dilution technique. The minimal biocidal concentration (MBC), was determined by subculturing all tubes from the MIC titrations without visible growth onto antibiotic-free agar medium. The subculture end points were read after 48 hours of incubation at 37°C, and the MBC was defined as the lowest concentration of antibiotic that did not allow growth of the organism on subculture.

Evaluation of the efficacy of EM49 was done in groups of CD-1 (Charles River) male mice, 16~18 g, that were infected experimentally. Evaluation in the *Streptococcus pyogenes* C203 model infection was as previously described by MIRAGLIA and BASCH[3]. The experimental *Escherichia coli* infection was produced by the intraperitoneal injection of a suitably diluted culture of *E. coli* Squibb Culture (SC) 8294, so that each mouse received 100 LD_{50} doses suspended in 1 ml of 5% hog gastric mucin. Treatment was given as a single dose within 1 hour after infection. The medicaments, in 5% gum acacia or in water, were administered by subcutaneous injection or by gavage. All

animals were observed for death or survival for 48 hours, then the experiment was terminated. The PD_{50} values were determined by the method of REED and MUENCH[4] or, in the occasional event of a survivor amongst the placebo-treated mice, by this method as modified by SCHOLER[5].

Results

Taxonomy

Microscopic examination of the organism that produces EM49 reveals a spore-forming bacillus that is gram-variable to gram-negative. The spores are located centrally to subcentrally within the sporangium. They are oval bodies with a thick, readily stainable wall. The sporangium is definitely swollen. Smears stained with 0.5 % basic fuchsin show faint encapsulation.

Macroscopic examination of the colonies on glucose-nutrient agar reveals glistening colonies with smooth to lobate edges. The colonies are adherent and very mucilaginous. The cells are motile and spread rapidly over the surface of the agar, particularly if the agar is moist. When grown in nutrient broth, the organism imparts a fair degree of turbidity to the broth and gives rise to a heavy sediment in the tube. A thick, mucilaginous pellicle is also formed, literally sealing the broth culture.

Neither indole nor acetylmethylcarbinol (VOGES-PROSKAUER test) is produced by the organism. Both starch and casein are strongly hydrolyzed by it. The organism does not form gas from carbohydrates, although it does grow and produce acid on inorganic media that contain glucose, sucrose and xylose as the sole carbon sources. The organism does not grow when incubated at 60~65°C. The properties just described have led to the identification of the organism as *Bacillus circulans*.

Activity *In Vitro*

Table 1 lists the susceptibility of various microorganisms to EM49. These data extend the spectrum given in the intial report[1] and demonstrate the unusual breadth of the spectrum *in vitro*: many gram-positive and gram-negative bacteria, as well as yeasts, fungi, and the protozoans *Trichomonas vaginalis* and *T. foetus*, are quite susceptible to this antibiotic. Especially interesting is the high order of the antipseudomonal activity, since this genus is generally refractory to the action of antibiotics. The higher order of activity against gram-negative than against gram-positive bacteria is also a distinguishing characteristic of this compound. Although not unique to EM49 it is, nevertheless, noteworthy.

Two of the characteristics noted, *i. e.*, the high degree of effectiveness against *Pseudomonas aeruginosa* and the greater activity against gram-negative than against gram-positive bacteria, are shared by polymyxin B. Thus, a comparison of the effectiveness of these two compounds *in vitro* was made, and the results are also shown in Table 1. It is evident from these data that of the two, EM49 has the broader spectrum of activity. Furthermore, when both compounds were compared for activity against a gram-positive bacterium, a yeast, or a fungus, EM49 was more potent than polymyxin B.

The action of EM49 on bacteria and on the yeast *Candida albicans* is clearly biocidal, and not biostatic. The ratio of the minimal biocidal concentration to the minimal inhibitory concentration is shown in Table 2. In each instance, the MBC/MIC ratio is 3 or less. More detailed studies were done to determine the rate at which bacteria are killed by EM49. Two gram-negative bacteria,

Table 1. A comparison of the antimicrobial spectra of EM49 and polymyxin B

Organism	Minimal inhibitory concentration (μg/ml)	
	EM49 hydrochloride	Polymyxin B sulfate
Staphylococcus aureus FDA 209P	5.5	25.0
Staphylococcus aureus SC* 2399	12.5	150.0
Staphylococcus aureus SC 2400	12.5	75.0
Staphylococcus epidermidis ATCC 12228	0.6	1.8
Staphylococcus epidermidis SC 9052	3.1	18.7
Streptococcus pyogenes C203	0.63	2.1
Streptococcus faecalis ATCC 10541	6.3	50.0
Diplococcus pneumoniae ATCC 6303	2.4	25.0
Bacillus cereus 17B**	18.7	>200.0
Bacillus cereus Philpot #1**	18.7	>200.0
Bacillus cereus Philpot #2**	18.7	>200.0
Bacillus cereus 69B**	37.5	>200.0
Corynebacterium minutissimum ATCC 23346	0.37	0.78
Corynebacterium minutissimum ATCC 23349	0.5	1.6
Escherichia coli ATCC 10536	0.78	0.05
Escherichia coli SC 8294	0.47	0.55
Escherichia coli SC 8517	0.25	0.19
Escherichia coli SC 8518	≤0.78	≤0.78
Serratia marcescens SC 1468	>100.0	>100.0
Klebsiella pneumoniae SC 8411	0.78	37.5
Klebsiella pneumoniae SC 8495	1.6	9.4
Pseudomonas aeruginosa SC 8327	<0.4	<0.4
Pseudomonas aeruginosa SC 8329	0.9	0.64
Pseudomonas aeruginosa SC 8754	0.4	0.2
Pseudomonas aeruginosa SC 8822	1.6	0.39
Pseudomonas aeruginosa SC 9108	0.4	1.2
Enterobacter cloacae SC 8405	0.9	0.8
Enterobacter cloacae SC 8415	1.8	0.8
Salmonella schottmuelleri SC 3850	0.8	0.05
Salmonella typhosa NIH #779	0.1	0.05
Proteus rettgeri SC 8217	100.0	>200.0
Proteus mirabilis SC 3855	150.0	>200.0
Bordetella bronchiseptica ATCC 19395	0.16	0.21
Vibrio parahemolyticus ATCC 17802	0.78	<0.09
Herella sp. SC 8333	1.0	0.5
Hemophilus suis ATCC 19417	0.6	0.3
Hemophilus influenzae ATCC 9333	1.0	0.06
Candida albicans SC 5314	9.4	66.7
Saccharomyces cerevisiae SC 1600	2.4	12.5
Aspergillus niger SC 2528	50.0	>200.0
Trichophyton mentagrophytes SC 2637	6.3	12.5
Microsporum canis SC 3767	6.3	25.0
Microsporum audouini SC 5282	6.3	12.5
Trichomonas vaginalis SC 8560	18.7	75.0
Trichomonas foetus SC 9644	37.5	37.5

* SC: from Squibb culture collection ** Clinical isolates from cases of bovine mastitis

P. aeruginosa SC 8329 and *E. coli* SC 8294, and the gram-positive bacterium, *Staphylococcus aureus* FDA 209P, were chosen for these studies. Viable-cell counts were made from samples taken at intervals during incubation of each of these bacteria at 37°C in the presence of EM49. The results are shown in Figs. 1, 2, and 3. A reduction in viable-cell count of these three organisms by a factor of at least 10^2 was accomplished within 0.5 hour by EM49 concentrations of 0.5~1.0, 10, and

Table 2. Bactericidal and candicidal activities of EM49 hydrochloride

Organism	EM49 hydrochloride		
	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC
Staphylococcus aureus FDA 209P	5.5	12.5	2
Pseudomonas aeruginosa SC 8329	0.9	3.1	3
Candida albicans SC 5314	9.4	25.0	3

Table 3. Antimicrobial activity of the components of EM49

Organism	Minimal inhibitory concentration (μg/ml)			
	EM49α*	EM49β	EM49γ	EM49δ
Staphylococcus aureus FDA 209P	12.5	6.3	3.1	2.4
Streptococcus pyogenes C203	1.6	0.8	0.4	0.4
Escherichia coli SC 2927	0.6	0.6	0.6	0.4
Pseudomonas aeruginosa SC 8329	0.8	0.8	0.4	1.2
Candida albicans SC 5314	9.4	9.4	6.3	4.7

* Fractions EM49α and β do not contain phenylalanine as part of their molecular structure, whereas EM49γ and EM49δ do. See ref. 2 for a discussion of the chemistry of these components.

Fig. 1. Bactericidal effect of EM49 HCl against *P. aeruginosa* SC 8329

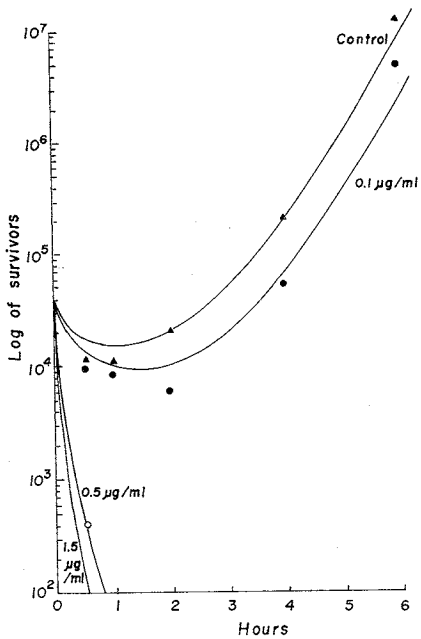


Fig. 2. Bactericidal effect of EM49 HCl against *E. coli* SC 8294

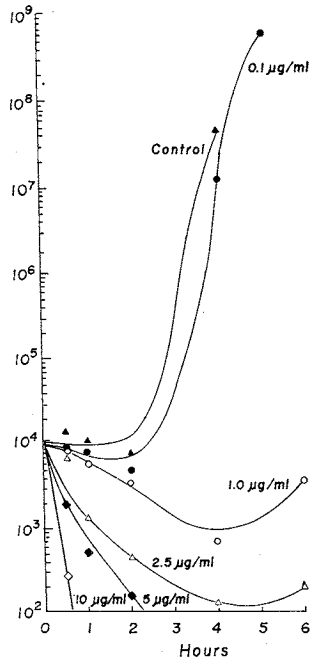
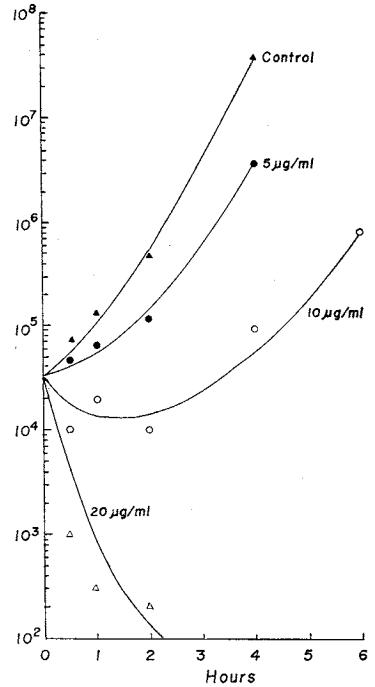


Fig. 3. Bactericidal effect of EM49 HCl against *S. aureus* FDA 209 P



20 μg/ml, respectively, thus demonstrating clearly the bactericidal nature of the antibiotic.

Attempts to develop resistance to EM49 *in vitro* were totally unsuccessful when *E. coli* SC 8294, *P. aeruginosa* SC 8329, or *S. aureus* FDA 209P were subcultured through the course of ten successive transfers in broth containing a range of antibiotic concentrations.

The importance of these findings is difficult to assess, because different mechanisms exist whereby microorganisms can become resistant to antibiotics. There is evidence to show that the mechanisms involved in the development of resistance in the laboratory can be different from those involved in the development of resistance encountered in the clinic[6,7].

As reported in the previous paper in this series[2], EM49 is a mixture of closely related peptides, differing by the presence or absence of phenylalanine and in the size of the fatty acid moiety. However, we determined that these structural differences have only a minor influence on the antimicrobial activity of these components, as shown by the data in Table 3.

In Vivo

EM49 protected mice against death from experimental infections of either *S. pyogenes* C203 or *E. coli* SC 8294 when it was given subcutaneously. The PD_{50} values obtained are shown in Table 4. However, when it was administered orally, EM49 failed to give any protection. This lack of effectiveness against systemic infections after oral administration of the drug is typical of peptide antibiotics, and is not peculiar to EM49.

Table 4. Activity of EM49 in experimental mouse infections

Infection*	Route of administration	PD_{50} (mg/kg)
Streptococcus pyogenes C203	Subcutaneous	110～130
	Oral	>300
Escherichia coli SC 8294	Subcutaneous	18.7
	Oral	>300

* See ref. 3 and the text for descriptions of the *Streptococcus pyogenes* C203 and *Escherichia coli* SC 8294 model infections respectively.

Because of its high degree of antipseudomonal activity, EM49 was tested for efficacy in an experimentally induced wound infection in mice. A surgical wound was made on the backs of mice, and the wound was then infected with *P. aeruginosa* SC 8822. The antibiotic, formulated in a suitable vehicle, *i.e.*, a cream base, was applied to the infected wound. Seventeen hours after the application of the antibiotic, counts of the viable bacteria surviving in the wound were made. EM49, at a concentration of 0.5% in the cream base vehicle, prevented the multiplication of *P. aeruginosa* SC 8822 in the wound by a significant degree, as compared with growth in the wounds of control animals that had received only the vehicle. The details of this model infection, as well as of other studies demonstrating the effectiveness of EM49 in topical infections, will be reported elsewhere.

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The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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