EM49, A NEW PEPTIDE ANTIBIOTIC

I. FERMENTATION, ISOLATION, AND PRELIMINARY CHARACTERIZATION

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EM 49 is a new basic peptide antibiotic complex produced by a strain of *Bacillus circulans*. When isolated as the hydrochloride, it has an approximate empirical formula of $C_{49\sim50}H_{91\sim98}N_{13}O_{10}Cl_4$. The antibiotic has broad-spectrum antibacterial activity and also displays considerable antifungal and antiprotozoal activities. EM 49 is not cross-resistant with other antibiotics, including those of a peptide nature.

During the past 3 decades of antibiotic screening, members of the genus *Bacillus* have proven to be the most fruitful of all in the order of Eubacteriales in the search for new antibiotics. More than 40 antibiotics have been described as being produced by members of this group[1]. We wish to report a new peptide antibiotic, EM 49, produced by a strain of *Bacillus circulans*. To our knowledge, only two other antibiotics have been reported to be produced by this species: polypeptin[2] and the aminoglycoside, butirosin[3]. Originally, circulin was also reported as a product of *B. circulans*[4], but the producing organism was subsequently reclassified as *Bacillus alvei*. (Personal communication from Dr. RUTH GORDON, Institute of Microbiology, Rutgers, The State University, to Mr. W. TREJO, Squibb Institute).

Production

The antibiotic is produced by a strain of *B. circulans* that has been deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A., under the accession number, ATCC 21,656. The bacterium is maintained on an agar-slant medium consisting of beef extract, 1.5 g; yeast extract, 3 g; peptone, 6 g; dextrose, 1 g; agar, 15 g; and distilled water to 1 liter. The growth was washed from the slants and used to inoculate 100-ml portions of medium contained in 500-ml, cotton-plugged Erlenmeyer flasks. The medium in these flasks had the following composition: soybean meal, 15 g; dehydrated mashed potato, 15 g; glucose, 50 g; $CoCl_2 \cdot 6H_2O$, 0.005 g; $CaCO_3$, 10 g; and distilled water to 1 liter. After 48 hours of incubation at 25°C on a rotary shaker, operating at 280 rpm with a 2-inch throw, a 5% (v/v) transfer was made to 500-ml, cotton-plugged Erlenmeyer flasks containing 100 ml of medium. The fermentation medium consisted of corn steep liquor, 6 g; $NH_4H_2PO_4$, 3 g; yeast extract, 2.5 g; dextrose, 10 g; $CaCO_3$, 2.5 g; Ucon LB 625, (Union Carbide, New York) 0.5 ml; and distilled water to 1 liter. The fermented broth was harvested after 144 hours of incubation at 25°C on the rotary shaker. The results of a typical flask fermentation are shown in Table 1.

Conventional twofold broth-dilution assays and paper disc-agar diffusion assays, with *Escherichia*

coli as the test organism, were used to follow the production of antibiotic as well as the degree of purification achieved during isolation. Thin-layer chromatography on silicic acid (Gelman ITLC, type SAF), with *n*-butanol-propionic acid-water (3 : 1 : 1 by volume) as the solvent system for development, was normally used to analyze samples. EM 49 has an R_f of 0.75 in this system.

Table 1. Production of EM 49 in shaken-flask fermentations

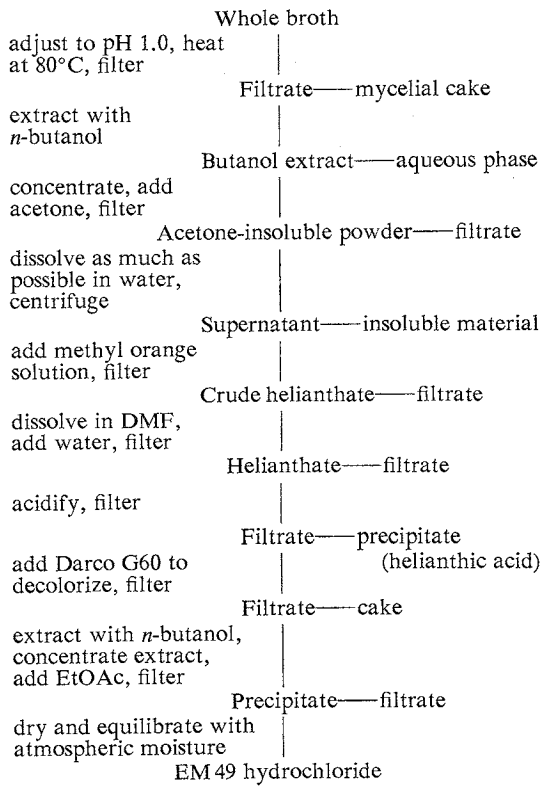
Fermentation time (days)	pH	Potency (dilution units/ml)*
2	5.9	960
5	6.7	960
6	6.8	1,920
7	6.8	3,840

* *Escherichia coli* SC 8294 was the test organism used in a twofold broth-dilution assay.

Isolation

The isolation of EM 49 is outlined in Chart 1. The viscous nature of the fermentation broth at harvest initially hindered isolation. However, this difficulty was overcome by acidification of the broth to pH 1.0 with concentrated hydrochloric acid and subsequent heating of the broth to 80°C and maintenance of that temperature for 30~60 minutes. This procedure resulted in a free-flowing liquid that showed negligible loss of antibiotic activity, and no discernible effect, chemical or biological, on the final isolated product. The treated broth was filtered, then extracted with *n*-butanol; the butanolic extract was concentrated *in vacuo*, below 40°C, to give a thick syrup. Addition of 10~15 volumes of acetone to the concentrate resulted in precipitation of the antibiotic. The precipitate was washed with acetone, then with ethyl acetate, and finally with ether. The material was dried *in vacuo* at 23°C, giving a light-tan powder that was usually 30~50 % pure EM 49.

Chart 1. Isolation and purification of EM 49 hydrochloride



Further purification was accomplished by preparation of the helianthate salt of the antibiotic. For example, 1 g of the acetone-insoluble powder was mixed with 20 ml of water; then any material that did not dissolve was removed by centrifugation. Addition to the supernatant liquid of a solution of 1 g of methyl orange in 15 ml of water and 5 ml of dimethylformamide (DMF) gave a precipitate of the helianthate. After filtration, the helianthate was washed with water, then redissolved in DMF and reprecipitated by the addition of water. After another filtration, the solid thus obtained was suspended in 10 ml of 0.36 N hydrochloric acid, resulting in the precipitation of the highly insoluble arenesulfonic acid and the concomitant liberation of the antibiotic into solution. The supernatant liquid was decolorized with charcoal (Darco G-60) and extracted with *n*-butanol. The extract was concentrated *in vacuo* below 40°C until the antibiotic had precipitated to form a thick slurry. After dilution of the slurry with

ethyl acetate, the solid material was separated and dried at 50°C and 0.02 mm, giving pure EM 49. The dried powder was equilibrated with atmospheric moisture, resulting in a preparation containing 5~12 % water.

For elemental analysis, a sample of the helianthate was reprecipitated from methanol-acetonitrile (2 : 1) and then from methanol, giving material that melted at 242~244°C (dec). Also, the *p*-phenylazobenzenesulfonate of EM 49 was prepared by adding *p*-phenylazobenzenesulfonic acid to an aqueous solution of EM 49 hydrochloride. The resulting precipitate crystallized when mixed with methanol. Several recrystallizations from methanol-acetonitrile (2 : 1) gave a product that decomposed at *ca.* 267°C when heated rapidly *in vacuo*.

Physical and Chemical Properties

EM 49 hydrochloride is a white powder that melts *in vacuo* with decomposition between 180 and 207°C. It dissolves freely in water and methanol. The hydrochloride salt can be converted to the free base by extracting an *n*-butanol solution of the antibiotic with portions of aqueous sodium hydroxide until chloride cannot be detected in the extract. Concentration of the butanolic solution to dryness gives an amorphous, hygroscopic solid that melts with decomposition at 263~265°C and has a neutral equivalent (adjusted to correspond to anhydrous material) of 255 by titration with perchloric acid.

Chromatographic mobilities of EM 49 in a number of solvent systems are shown in Table 2. The antibiotic was detected by bioautography against *Escherichia coli*. Ninhydrin can also be used for detection.

The molecular weight of EM 49 was determined by ultracentrifugation[5]. The hydrochloride forms polymolecular aggregates in solution. For example, molecular weights of 1,250 and 2,520 were observed in ethanol, whereas the free base in methanol gave a single value of 1,080±120.

Elemental analyses are shown in Table 3. The equivalent weight and the molecular weight of the free base indicate that the antibiotic is a tetraacidic base. With the exception of oxygen, no elements except those shown in Table 3 were found in greater than trace amounts. Based on these data, the approximate empirical formula of EM49 hydrochloride is $C_{49\sim50}H_{91\sim98}N_{13}O_{10}Cl_4$.

The infrared absorption spectrum of the hydrochloride (Fig. 1) is typical of a peptide. Hydrolysis of the antibiotic in 6 N hydrochloric acid at 110°C for 17 hours gave a mixture of amino acids identified by paper chromatography and by quantitative amino acid analysis as 2, 4-

Table 2. Paper chromatography* of EM 49

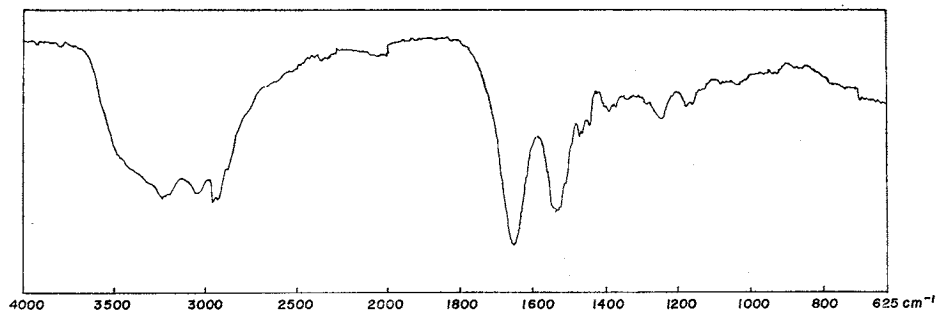
Solvent system**	R_f
n-Butanol, acetic acid, water (4 : 1 : 5)	0.71
n-Propanol, *n*-butanol, water (2 : 3 : 4)	0.57
Chloroform, methanol, water (5 : 4 : 2)	0.38
Chloroform, methanol, 1 % acetic acid (5 : 4 : 2)	0.35
Chloroform, methanol, 0.5 N NH_4OH (5 : 4 : 2)	0.91

* Whatman # 1 paper, chromatographic grade
** By volume

Table 3. Elemental analyses

Compound	% Found				
	C	H	N	Cl	S
EM 49 free base	58.40	8.60	17.84	—	—
EM 49 hydrochloride	50.80	7.96	15.18	12.01	—
EM 49 helianthate	56.18	6.92	15.60	—	5.73
EM 49 *p*-Phenylazo-benzenesulfonate	56.25	6.40	14.40	—	6.10

Fig. 1. Infrared absorption spectrum of EM 49 hydrochloride in KBr



diaminobutyric acid (five residues per molecule), leucine (*ca.* two and one-half residues) and phenylalanine (*ca.* one-half residues). It was inferred from these data that the antibiotic was not homogeneous. Acid hydrolysis also produced a mixture of fatty acids that will be discussed in another publication[6]. The ultraviolet absorption spectrum of EM 49 in water showed end absorption ($E^{1\%}_{1cm}$ 109~122 at 209 nm) and a series of very weak maxima between 245 and 270 nm, due to the phenylalanine residue. The specific rotation was $[\alpha]^{23}_{D}$—20.5 (*c* 1, DMSO).

Chemically, EM 49 is most closely related to the polymyxin group of antibiotics. However, EM 49 is a complex of octapeptide antibiotics

Table 4. Antimicrobial spectrum of EM 49 hydrochloride

Organism	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus FDA 209P	5.5
Streptococcus pyogenes C203	0.6
Bacillus subtilis ATCC 6633	0.6
Escherichia coli SC 8294*	0.5
Pseudomonas aeruginosa SC 8329*	0.9
Serratia marcescens SC 1468*	>100.0
Candida albicans SC 5314*	9.4
Trichophyton mentagrophytes SC 2637*	6.3
Trichomonas vaginalis SC 8560*	18.7

* Squibb culture collection

Table 5. Activity of EM 49 hydrochloride against antibiotic-resistant variants of *Staphylococcus aureus* FDA 209P *in vitro*

Staphylococcus aureus strain number	Minimum inhibitory concentration (μg/ml)
FDA 209P	5.5
SC 2961*	12.5
SC 2664	12.5
SC 2661	3.1
SC 2957	18.7
SC 3538	12.5

* Squibb culture collection.
SC 2961 is resistant to thiostrepton, erythromycin, oleandomycin, methymycin and carbomycin.
SC 2664 is resistant to streptomycin, neomycin, and tetracyclines.
SC 2661 is resistant to thiostrepton.
SC 2957 is resistant to actinomycin, chloramphenicol, and bacitracin.
SC 3538 is resistant to penicillins and ristocetin.

Table 6. Activity of EM 49 hydrochloride against polymyxin B-resistant variants of *Escherichia coli*

Organism (SC Number*)	Minimum inhibitory concentration (μg/ml)	
	EM 49 hydrochloride	Polymyxin B sulfate
SC 8599**	2.3	0.8
SC 8600	0.13	>50.0
SC 9251***	1.65	1.17
SC 9252	0.3	100.0
SC 9253	<0.1	>200.0

* Squibb culture collection
** *Escherichia coli* SC 8599 is the polymyxin B-sensitive parent from which the resistant variant SC 8600 was derived.
*** *Escherichia coli* SC 9251 is the polymyxin B-sensitive parent from which the resistant variants SC 9252 and SC 9253 were derived.

lacking threonine, whereas the polymyxins are all decapeptides that release threonine upon acid hydrolysis.

Biological Properties

EM49 possesses broad-spectrum antibacterial activity as well as substantial activities against yeasts, fungi and protozoa. The antimicrobial spectrum *in vitro*, obtained by conventional twofold broth-dilution assay, is shown in Table 4. The antibiotic is not cross-resistant with a number of other antibiotics representing different chemical types. This absence of cross-resistance is demonstrated (Table 5) by the effectiveness of EM 49 against a number of variants of *Staphylococcus aureus* FDA 209P that are resistant to such antibiotics as penicillins, macrolides, aminoglycosides, tetracyclines, peptides and chloramphenicol. Especially noteworthy is the absence of cross-resistance between EM 49 and polymyxin B (Table 6); indeed, an indication of inverse sensitivity can be inferred from the data. Polymyxin B was chosen for comparison because of its chemical similarities to EM 49[6], because both antibiotics are produced by a species of *Bacillus*, and because both are more active against *Escherichia coli* than against *Staphylococcus aureus*.

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