ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1975, p. 507-516

Vol. 7, No. 5
Printed in U.S.A.

Antimicrobial Activities of 81.723 hfu, a New Pleuromutilin Derivative

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Received for publication 31 December 1974

The new pleuromutilin derivative 81.723 hfu is extremely active against gram-positive organisms such as streptococci, staphylococci, and against mycoplasmas. A number of *Shigella*, *Klebsiella*, and *Escherichia coli* strains were also found to be quite susceptible to this new agent, whereas other gram-negative organisms like *Pseudomonas aeruginosa*, *Proteus* species, and *Alcaligenes faecalis* proved to be naturally resistant to 81.723 hfu. The new compound acts bacteriostatically. Bactericidal effects have been observed only at concentrations which are 100-fold higher than the minimal inhibitory concentrations. The new antibiotic is well tolerated in all animal species tested so far and has been successfully used in the treatment of experimental infections with gram-positive organisms and with mycoplasmas in mice and rats. Resistance against this new compound arose gradually in all microorganisms investigated. It is noteworthy that the rate at which resistance against 81.723 hfu emerged in mycoplasmas (*Mycoplasma gallisepticum* and *Mycoplasma hyorhinis*) was significantly slower than the corresponding rate at which resistance against tylosin tartrate appeared. Mycoplasma strains which became insensitive to 81.723 hfu were also resistant to tylosin tartrate, whereas mycoplasmas which developed resistance against tylosin tartrate, although less sensitive to 81.723 hfu than wild-type strains, were still eliminated by this drug. In a strain of *Klebsiella pneumoniae*, complete cross-resistance was observed between the pleuromutilin derivative on one hand and lincomycin and erythromycin on the other. Modest degrees of cross-resistance were also observed with chloramphenicol. However, it appears unlikely that the latter phenomenon is sufficiently pronounced to affect treatment with either antibiotic.

Cultures of the basidomycete *Pleurotus mutilis* contain a substance with a modest degree of in vitro activity against gram-positive bacteria which was called pleuromutilin (6). This compound was shown to be a diterpene antibiotic and to have the structure depicted in Fig. 1 (1, 9). Experiments which will be reported elsewhere demonstrated that the antibacterial activity of pleuromutilin can be greatly enhanced if the glycolic side chain in position 14 of the molecule is replaced by other acyl residues. Following this general strategy, a large number of pleuromutilin derivatives, all of which exhibited considerable antimicrobial activity in vitro and in vivo, were synthesized. From this series, 14-deoxy-14-[(2-diethylaminoethyl)-mercapto-acetoxy]-mutilin was selected for further study on the basis of the following criteria: (i) availability as a crystalline water-soluble salt (hydrogen fumarate); (ii) outstanding activity against gram-positive bacteria and mycoplasma strains in vitro; (iii) good efficacy against experimental animal infections due to gram-positive bacteria and mycoplasmas; and (iv) low to moderate acute toxicity in several animal species.

The purpose of this paper is to describe the new compound's antimicrobial and chemotherapeutic potential.

MATERIALS AND METHODS

Antibiotics. Compound 81.723 hfu was synthesized by procedures which will be described elsewhere (manuscript in preparation). Erythromycin was a gift of Schering AG, Berlin. Lincomycin and spectinomycin came from the Upjohn Co., Kalamazoo, Mich. Tetracycline-hydrochloride was obtained commercially.

Organisms. If not otherwise stated, bacteria were isolated from the Tierärztliche Hochschule, Vienna, or from this institute. The isolated organisms were classified according to the taxonomic criteria laid down in *Bergey's Manual of Determinative Bacteriology* (3). Most mycoplasmas were obtained from the American Type Culture Collection. *Mycoplasma gallisepticum* (FS 9) was a gift from C. O. Baughn, The

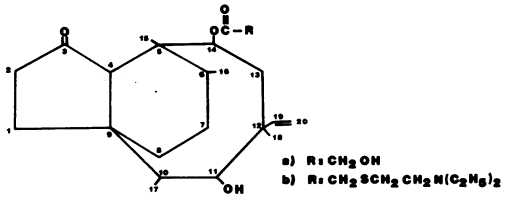

FIG. 1. *Structure of pleuromutilin and its derivative 81.723 hfu. (a) Pleuromutilin; (b) 14-deoxy-14-[(2-diethylaminoethyl)-mercaptoacetoxy]- mutilin. The hydrogen fumarate moiety was omitted from this figure.*

Squibb Institute of Medical Research, New Brunswick, N.J., *M. hyopneumoniae* was obtained from W. P. Switzer, Iowa State University, and *M. pneumoniae* and *M. synoviae* were obtained from W. Bredt, Würzburg, and E. A. Freundt, Aarhus, respectively. All mycoplasms strains were typed and classified according to the standards made available to us by the FAO/WHO International Reference Centre for Animal Mycoplasmas, University of Aarhus, Aarhus, Denmark.

Media. Most bacterial organisms were grown and tested for their susceptbility to antibiotics on Trypticase soy broth or 1.3% Trypticase agar (Baltimore Biological Laboratory). For culturing *Haemophilus influenzae*, Fieldes enrichment medium was added to Trypticase soy broth to give a final concentration of 5% (vol/vol). Clostridia were grown and assayed in anaerobic agar (BBL). Minimal inhibitory concentration (MIC) for mycoplasmas were determined on mycoplasma broth base or on agar base (Oxoid). *M. hyopneumoniae* was grown and assayed as described by Slavik and Switzer (13), whereas growth conditions for *M. synoviae* were chosen according to Frey at el. (4).

Animals. National Medical Research Institute (NMRI) mice weighing 17 to 20 g were obtained from Gassner Animal Farms, Germany. Female Sprague-Dawley rats (specific pathogen free) weighing 80 to 100 g were provided by Wiga, Sulzfeld, Germany. Hubbard broiler chicken weighing 370 to 500 g, turkeys (280 to 400 g), and pigeons came from a commercial breeding station and were free of mycoplasmas.

In vitro activity. MICs for bacteria and mycoplasmas were determined by serial twofold dilution tests in the appropriate media.

Development of resistance in vitro. The development of bacterial resistance against antibiotics was studied by growing the respective organisms in the presence of various concentrations of the drug against which resistance was to be generated. From the tube containing the highest concentration of antibiotic which still allowed normal or nearly normal growth, successive transfers were made every 48 h into the next series of tubes containing the same or higher concentrations of the antibiotic. The same technique was used for the generation of resistance of mycoplasmas.

Bacteriostatic versus bactericidal activity in vitro. Samples (1.2 ml) of Trypticase soy broth containing compound 81.723 hfu in concentrations increasing geometrically from 0.039 to 10 μg/ml were inoculated with 10^3 organisms of *Staphylococcus aureus* SG 511 and incubated at 37 C for 18 h. Control tubes contained no antibiotic. Aliquots (0.1 ml) from these cultures were plated on blood agar. After 24 h of incubation the number of colonies on each plate was counted. This experiment was carried out at pH 7.3 and 8.0.

Protein biosynthesis. *Klebsiella pneumoniae* wild type and mutants resistant against 81.723 hfu were grown in Trypticase soy broth to half-logarithmic phase. The cells were then harvested, and the ribosomes were isolated according to Nirenberg (10). Twenty absorbancy units of these ribosomes at 260 nm were then introduced into a cell-free, protein-synthesizing system containing (in volumes of 0.25 ml): 500 μg of a 100,000 × *g* supernatant from *Escherichia coli* K-12 as determined by the method of Lowry et al. (8), 3.5 absorbancy units of polyuridylic acid at 260 nm, 16 mM $MgCl_2$, 0.2 mM guanosine 5'-triphosphate, and 0.2 μCi of [^{14}C]phenylalanine. The antibiotic was introduced to give final concentrations between 10^{-9} and 10^{-2} M. Incubations which lasted 15 min at 37 C were terminated by precipitation with 10% (wt/vol) ice-cold trichloroacetic acid. After centrifugation, the precipitates were resuspended in 10% trichloroacetic acid and kept at 90 C for 30 min. They were then centrifuged and washed four times with cold trichloroacetic acid. Subsequently, the samples were applied to nitrocellulose filters for scintillation counting. The radioactivity of samples incubated in the absence of the inhibitor were called 100% and the data obtained in the presence of the anitbiotic were plotted as percentage of controls.

Acute toxicity. Acute toxicity experiments were performed in four animal species: female mice, male chickens, 4-week-old male and female pigeons, and 3-week-old male and female turkeys. 81,723 hfu was administered to these animals as a single dose either perorally, subcutaneously, or intramuscularly. There were six dosage groups for each animal species and each route of administration. Each group consisted of at least 10 animals. After administration of the drug, the animals were observed continuously for 5 h and daily for a subsequent period of 2 weeks. The mortality in each group was recorded, and dose response curves were drawn for each experimental group. The mean lethal dose (LD) values for 5, 50, and 95% of the animals were then computed by probit analysis according to Bliss (2).

Experimental infections of mice. Animals were infected intraperitoneally with 0.3 ml of bacterial suspensions of *S. aureus* ATCC 10930, β-hemolytic *Streptococcus* A, or *Streptococcus aronson*. *S. aureus* ATCC 10930 was grown on Trypticase soy broth for 18 h. At this time the cultures contained approximately 10^8 organisms/ml. From this culture appropriate dilutions containing 5% (vol/vol) dimethyl sulfoxide were prepared for inoculation. The challenge dose in each experiment was three times the LD_{50} or 6×10^7 organisms. β-Hemolytic *Streptococcus* group A was grown in nutrient broth (BBL) containing 5% (vol/vol) horse serum. Eighteen-hour cultures containing 5×10^7 organisms/ml and 10^{-4} to 10^{-7} dilutions of this

culture were prepared with 5% (vol/vol) dimethyl sulfoxide in saline. The challenge dose was 5 LD_{50}. Cultures of *Streptococcus aronson* group D were grown and diluted as described for β-hemolytic streptococci. The LD_{50} was found to be three or four organisms per mouse. Ten to 20 LD_{50} were used as challenge dose. Infected mice were treated with a single dose of each antibiotic immediately after challenge. The antibiotics were dissolved in saline and administered orally by stomach tube or injected subcutaneously. Deaths due to infection were recorded daily and the mean effective doses (ED_{50}) (milligrams per kilogram) were determined after 10 days by the method of Reed and Muench (11).

Infection of rats with M. arthritidis. Details of this experimental infection have been published previously (12). Female Sprague-Dawley rats weighing 80 to 100 g received 1 ml of a culture of *M. arthritidis* (10^9 organisms/ml) plus 1 ml of a 5% (wt/vol) kieselguhr suspension intraperitoneally. The size of the inoculum corresponded to three times the infectious dose necessary to produce symptoms in 95% of the animals (ED_{95}). Criteria of infection were occasional death, paralysis, and arthritis. The number of animals showing signs of infection was used for quantitative evaluation of the test, whereas the severity of the symptoms was not taken into account. 81.723 hfu, when applied subcutaneously, was given in three single doses 1, 24, and 48 h after infection. When the compound was administered orally, the total dose was divided into five single doses which were applied by stomach tube 1, 6, 24, 30, and 48 h after infection. ED_{50} values were calculated on the basis of the total doses according to standard procedures (11).

RESULTS

In vitro activity of 81.723 hfu. 81.723 hfu displays strong antibacterial effects in vitro against most gram-positive organisms tested (Table 1). The MICs against staphylococci were generally one order of magnitude lower than the corresponding values for tylosin tartrate and considerably lower than the values for tetracycline. The new pleuromutilin derivative was clearly less effective against most gram-negative organisms than tetracycline-hydrochloride. However, five out of six tested strains of various *Klebsiella* species and 10 of 12 strains of *E. coli*, as well as *Pasteurella multocida* and *Shigella flexneri*, were susceptible to compound 81.723 hfu in concentrations less than 12.5 μg/ml.

The in vitro effectiveness of 81.723 hfu against a number of mycoplasma strains pathogenic for animals was clearly superior to that of tetracycline and also better than that of tylosin tartrate (Table 2).

The MICs determined for 81.723 hfu proved to be constant over a large range of inoculum sizes for all organisms tested. Only inocula greater than 10^5 organisms/ml resulted in a significant rise in the MIC values (data not shown). Other parameters influencing the in vitro efficacy of 81.723 hfu were also studied. The MIC values determined for 81.723 hfu in brain heart infusion broth (BBL) were usually one-third of the corresponding values measured in nutrient broth, Trypticase soy broth, or antibiotic assay broth. 81.723 hfu displayed greatest activity at slightly alkaline pH values. A drastic reduction in antibacterial activity was observed at pH levels below the physiological range (Table 3). This finding is analogous to the loss of antibacterial activity observed with macrolide antibiotics in an acid environment.

To decide whether compound 81.723 hfu displays a bacteriostatic or bactericidal action, cultures of *S. aureus* SG 511 were incubated in the presence of 81.723 hfu in concentrations ranging from 0.039 (MIC at pH 8.0) to 10 μg/ml. The number of bacteria remaining viable after exposure to compound 81.723 hfu was then determined by subculturing 0.1-ml aliquots from each tube on blood agar plates and counting the number of colonies. The results of this experiment (Table 4) indicate that compound 81.723 hfu acts bacteriostatically over a wide range of concentrations. Only concentrations of the antibiotic which were 50- to 100-fold higher than the MIC values resulted in bactericidal effects.

Development of resistance against compound 81.723 hfu. Resistance against 81.723 hfu in several bacterial organisms emerged at a rate which was comparable to the acquisition of resistance against a number of other antibiotics by the same organisms. Results presented in Table 5 were obtained by incubating the test strains on 1.4% Trypticase soy agar in the presence of increasing concentrations of the respective antibiotic and by passaging those colonies which had grown on the highest concentration of antibiotic. In our experiments, the increase in resistance against 81.723 hfu occurred gradually and followed the multiple-step pattern. In mycoplasmas, resistance against 81.723 hfu appeared to emerge even more slowly. Figure 2 illustrates the generation of resistance against tylosin tartrate and 81.723 hfu in *M. gallisepticum* and *M. hyorhinis*. Whereas the sensitivity of *M. gallisepticum* against tylosin tartrate begins to decrease significantly between the third and fifth passage and resistance against this drug has reached very high values after 10 passages, there is hardly any increment in resistance against 81.723 hfu during the first 10 passages. After 20 passages the MIC of compound 81.723 hfu for *M. gallisepticum* amounts to 12.5 μg/ml as compared to an MIC for tylosin tartrate of almost 5,000 μg/ml.

TABLE 1. *In vitro activity of 81.723 hfu, tetracycline-hydrochloride, and tylosin tartrate against gram-positive and gram-negative bacteria*[a]

Test organism	MIC (μg/ml)		
	81.723 hfu	Tetracycline-hydrochloride	Tylosin tartrate
S. aureus SG 511	0.031–0.039	0.097–0.156	0.39–0.625
S. aureus Smith	0.015–0.019	0.097–0.156	0.62–0.78
S. aureus ATCC 10390	0.062–0.078	0.097–0.156	0.78–1.25
S. aureus 209 P	0.031–0.039	0.097–0.156	0.39–0.625
S. aureus ATCC 14154 (tetracycline and penicillin resistant)	0.0125–0.015	100	0.78–1.25
S. aureus (tetracycline and penicillin resistant)	0.0078–0.0125	100	0.39–0.625
S. aureus (tetracycline resistant)	0.062–0.078	100	0.78–1.25
S. aureus (tetracycline resistant)	0.015–0.019	>100	0.78–1.25
S. aureus (tetracycline resistant)	0.031–0.039	100	0.78–1.25
S. albus	0.125–0.156	0.156–0.195	0.078–0.097
Micrococcus Oxford	0.015–0.019	0.078–0.097	0.19–0.31
β-Hemolytic *Streptococcus*, Lancefield group A	0.031–0.039	0.19–0.31	0.097–0.156
Streptococcus aronson	0.019–0.031	0.39–0.625	0.156–0.195
Streptococcus faecalis	0.62–0.78	0.097–0.156	0.39–0.625
Streptococcus faecalis	25	0.39–0.62	1.25–1.56
Streptococcus pyogenes ATCC 8668	0.039–0.062	0.195–0.312	0.195–0.312
Streptococcus lactis	25	0.195–0.312	0.78–1.25
Bacillus subtilis ATCC 6633	10.0–12.5	0.156–0.195	0.19–0.31
B. cereus ATCC 9634	25	≥0.039	0.31–0.39
Clostridium perfringens	0.625–0.78	1.56–2.5	0.625–0.78
C. perfringens	0.39–0.625	1.56–2.5	0.625–0.78
Sarcina lutea ATCC 9341	0.031–0.078	0.62–0.78	0.19–0.31
Corynebacterium equi	50	2.5–3.12	25
Erysipelothrix	2.5–3.12	0.78–1.25	5.0–6.25
Listeria monocytogenes	3.12–5.0	0.625–0.78	0.625–0.78
Pseudomonas sp.	>100	250	>100
E. coli, unclassified	10.0–12.5	0.78–1.25	>100
E. coli, unclassified	25	0.78–1.25	>100
E. coli D10	10.00–12.5	0.78–1.25	>100
E. coli, unclassified	12.5	0.78–1.25	>100
E. coli O4	10.0–12.5	0.78–1.25	>100
E. coli O4	25	0.78–1.25	>100
E. coli O25	12.5		>100
E. coli O149	12.5		>100
E. coli O8	10.0–12.5		>100
E. coli O147	0.625–0.78	0.39–0.62	50
E. coli O9	2.5–3.12		50
E. coli O101	6.25–10.0		>100
Salmonella typhimurium	25	1.25–1.56	>100
Proteus morganii	>100	2.5–3.12	>100
Shigella flexneri	3.12–5.0	0.39–0.62	>100
Aerobacter aerogenes	100	2.5–3.12	>100
Alcaligenes faecalis	>100	1.25–1.56	>100
K. pneumoniae	0.625–0.78	0.39–0.62	100
K. pneumoniae ATCC 10031	1.25–2.5	0.78–1.25	>100
K. sp.	100	3.12–3.9	>100
K. rhinoskleromatis	6.25–10.0	0.39–0.62	100
K. rhinoskleromatis	6.25–10.0		100
K. rhinoskleromatis	6.25–10.0		100
Pasteurella multocida	5.0–6.25	≥0.039	10.0–12.5
Serratia marcescens	25	25.0–31.2	>100
Neisseria perflava	1.56–2.5	25	1.25–1.56
Haemophilus influenzae	0.625–0.78	5.0–6.25	0.78–1.25

[a] The three compounds were tested at pH values optimal for their in vitro activity: 81.723 hfu, pH 8.0; tetracycline-hydrochloride, pH 6.6; tylosin-tartrate, pH 7.3.

The *M. gallisepticum* strain which had become resistant to concentrations of 81.723 hfu below 12.5 μg/ml proved to be fully resistant against tylosin tartrate with MICs well above 1,000 μg/ml. Conversely, the tylosin tartrate-resistant strain had become less sensitive to 81.723 hfu, the corresponding MIC value being 2.5 μg/ml. For most practical purposes, however, this strain could still be considered sensitive to the pleuromutilin derivative. The rates at which resistance to 81.723 hfu and to tylosin tartrate appear in *M. hyorhinis* are almost identical to the corresponding rates observed with *M. gallisepticum* (Fig. 2B). In subsequent experiments, bacterial strains which had been made resistant to 81.723 hfu were examined for their sensitivity to chloramphenicol, lincomycin, erythromycin, and spectinomycin. A strain of *K. pneumoniae* resistant to 81.723 hfu displayed cross-resistance to lincomycin and erythromycin but showed only a modest loss of sensitivity to chloramphenicol and spectinomycin (Table 6).

TABLE 2. *In vitro susceptibility of various mycoplasmas to 81.723 hfu and standard antibiotics*

Species	MIC (μg/ml)[a]		
	Tetracycline-hydrochloride	81.723 hfu	Tylosin tartrate
Mycoplasma arthritidis PG 6, ATCC 19611	50	0.15	3.1
M. arthritidis[b]	6.25	0.625	0.312
M. bovigenitalium PG11, ATCC 19852	0.31	0.06	0.12
M. bovimastitidis ATCC 25025	100	1.25	1.25
M. bovirhinis PG 34, ATCC 19884	100	1.25	1.25
M. canis PG 14, ATCC 19525	12.5	0.15	1.25
M. felis ATCC 23391	12.5	0.039	2.5
M. fermentans PG 18, ATCC 19989	12.5	0.15	0.31
M. gallisepticum PG 31, ATCC 19610	0.62	0.0062	0.031
M. gallisepticum S 6, ATCC 15302	1.25	0.0078	0.062
M. gallisepticum FS 9	0.62	0.0039	0.062
Acholeplasma granularum ATCC 19168	62.5	0.62	6.25
M. hominis H 27, ATCC 15488	6.25	0.012	0.625
M. hyopneumoniae S 11/P 25	0.31	0.031	0.031
M. hyorhinis	5	0.25	1
M. hyorhinis	0.62	0.039	1.25
M. hyorhinis	2.5	0.31	2.5
M. hyorhinis BTS 7, ATCC 17981	1.25	0.156	1.25
M. hyosynoviae	10	0.05	0.062
Acholeplasma laidlawii PG 8, ATCC 23206	250	6.25	12.5
A. laidlawii PG 9	250	3.12	12.5
M. meleagridis ATCC 25294	5	0.25	0.5
M. pneumoniae FH	5	0.031	0.031
M. pulmonis ATCC 19612	12.5	0.31	0.62
M. synoviae	0.15	0.031	0.062

[a] Inoculum sizes were 5×10^6 colony-forming units per 2-ml assays.

[b] Strain isolated from a rat with labyrinthitis and classified as *M. arthritidis* by this laboratory and by B. W. Andrews, Mycoplasma Reference Laboratory, London.

TABLE 3. *Antibacterial in vitro activity of 81.723 hfu as a function of pH*

Strains tested	MIC (μg/ml)						
	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5
Micrococcus Oxford	2.5	1.0–1.25	0.25–0.312	0.156–0.25	0.062–0.078	0.009–0.015	0.0078–0.0125
S. aureus ATCC 10390	≥5.0	≥5.0	1.0–1.25	0.5–0.625	0.156–0.25	0.078–0.125	0.062–0.1
β-Hemolytic *Streptococcus*		0.312–0.5	0.312–0.5	0.156–0.25	0.078–0.125	0.019–0.031	0.0078–0.0125
E. coli O4	>100	>100	>100	100	50	25	10–12.5
K. pneumoniae	50	50	≥12.5	5.0–12.5	2.5–3.12	0.78–1.25	0.62–0.78
Shigella flexneri	>100	>100	>100	100	25	6.25–10	3.12–5.0

TABLE 4. *Viable organisms of S. aureus SG 511 in cultures exposed to various concentrations of compound 81.723 hfu*

pH value	Concn of 81.723 hfu (μg/ml)									
	0	0.039[a]	0.078	0.156[b]	0.312	0.625	1.25	2.5	5.0	10.0
7.3	+++[c]	+++	+++	+++	++	+	+	+	−	−
8.0	+++	+++	+++	+++	+	+	+	−	−	−

[a] MIC at pH 8.0.
[b] MIC at pH 7.3.
[c] +++, at least 10^3 organisms/ml; ++, 10^2 to 10^3 organisms/ml; +, less than 10^2 organisms/ml; −, sterile culture.

TABLE 5. *Generation of resistance to 81.723 hfu and five standard antibiotics in two strains of S. aureus and in K. pneumoniae*

Antibiotic	Strain	MIC (μg/ml)		Increase in resistance[a] (-fold)	No. of passages
		Wild type	Mutant		
81.723 hfu	*S. aureus* SG 511	0.35	60	172	7
	S. aureus ATCC 10390	0.35	125	358	7
	K. pneumoniae ATCC 10031	4.0	500	125	4
Lincomycin	*S. aureus* SG 511	0.35	25	71	6
	S. aureus ATCC 10390	0.5	15	30	6
	K. pneumoniae ATCC 10031	40	5,000	125	3
Chloramphenicol	*S. aureus* SG 511	4	125	31	8
	S. aureus ATCC 10390	2.8	50	18	8
	K. pneumoniae ATCC 10031	1	1,000	1,000	7
Erythromycin	*S. aureus* SG511	0.1	60	600	6
	S. aureus ATCC 10390	0.1	50	500	5
	K. pneumoniae ATCC 10031	2.0	500	250	3
Spectinomycin	*S. aureus* SG 511	3.5	1,000	330	2
	S. aureus ATCC 10390	10	1,000	100	2
	K. pneumoniae ATCC 10031	2.5	5,000	2,000	5

[a] Factor indicating the rise in MIC values of resistant strains over the MIC values of the corresponding wild types.

Correspondingly, mutants of the same *Klebsiella* strain which had been selected for their resistance to lincomycin and erythromycin also proved to be resistant to the pleuromutilin derivative.

A less characteristic pattern of cross-resistance was found to exist in two species of *S. aureus*. Mutants of *S. aureus* SG 511 and *S. aureus* ATCC 10390 which had acquired resistance to 81.723 hfu only showed a modest loss of sensitivity to lincomycin and almost no alteration in their sensitivity to erythromycin. Representatives of these two strains which had developed resistance to lincomycin displayed only a three- to sixfold increase in their resistance to 81.723 hfu. Erythromycin- and spectinomycin-resistant mutants of these two strains remained fully susceptible to the pleuromutilin derivative.

In a recent publication from this laboratory, Hodgin and Högenauer (5) demonstrated an inhibition of bacterial protein synthesis to be the biochemical basis for the antibacterial action of pleuromutilin derivatives. It was shown by these authors that pleuromutilin derivatives prevent the binding of aminoacyl-transfer ribonucleic acid to the ribosomal A site if a peptidyl transfer ribonucleic acid or an initiator transfer ribonucleic acid occupies the ribosomal P site (5). This finding, as well as the occurrence of cross-resistance with erythromycin and lincomycin in mutants of *K. pneumoniae* described above and to chloramphenicol in other bacterial mutants (14), raised the possibility that an

alteration of the bacterial ribosome might represent the biochemical basis for resistance to 81.723 hfu. This hypothesis was put to experimental trial by isolating ribosomes from a sensitive wild type of *K. pneumoniae* and from six mutants from this organism which had become resistant against 81.723 hfu. These ribosomes were then introduced into a classical cell-free system for protein synthesis, in which all other cellular components necessary for the formation of polyphenylalanine coded by polyuridylic acid were derived from *E. coli*. It was found that the concentrations of 81.723 hfu necessary to produce a 50% inhibition of protein synthesis in those assays which contained ribosomes from resistant strains was higher by three to four orders of magnitude than the concentration of the antibiotic which had the same effect in the control system containing ribosomes from the sensitive wild-type strain (Fig. 3). It was concluded from these experiments that an alteration of the bacterial ribosome provides at least one mechanism by which bacteria can become resistant to 81.723 hfu.

In vivo experiments with compound 81.723 hfu. A summary of data relating to the acute toxicity of the new antibiotic in four animal species is given in Table 7.

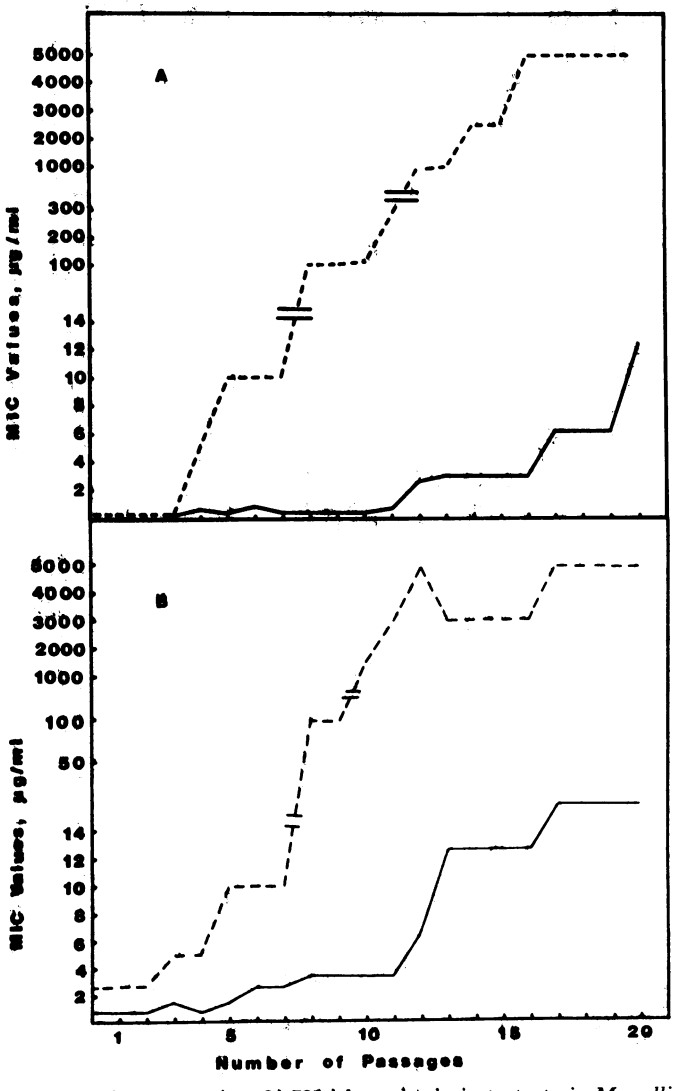

FIG. 2. *Development of resistance against 81.723 hfu and tylosin tartrate in M. gallisepticum (A) and M. hyorhinis (B). Dotted lines, MIC values of tylosin tartrate; solid lines, MIC values of 81.723 hfu.*

TABLE 6. *Cross-resistance of 81.723 hfu and various antibiotics in K. pneumoniae ATCC 10031*

Resistance produced against	Increase in MIC (-fold)	Cross-resistance[a] against				
		81.723 hfu	Lincomycin	Chloram-phenicol	Erythro-mycin	Spectino-mycin
81.723 hfu	>100	–	25	3	>50	1
Lincomycin	125	125	–	125	>250	12
Chloramphenicol	1,000	15	>12	–	1	1
Erythromycin	250	30	6	2.5	–	1
Spectinomycin	2,000	15	200	1	>250	–

[a] Values indicate x-fold increase in the minimal inhibitory concentrations observed for the wild type.

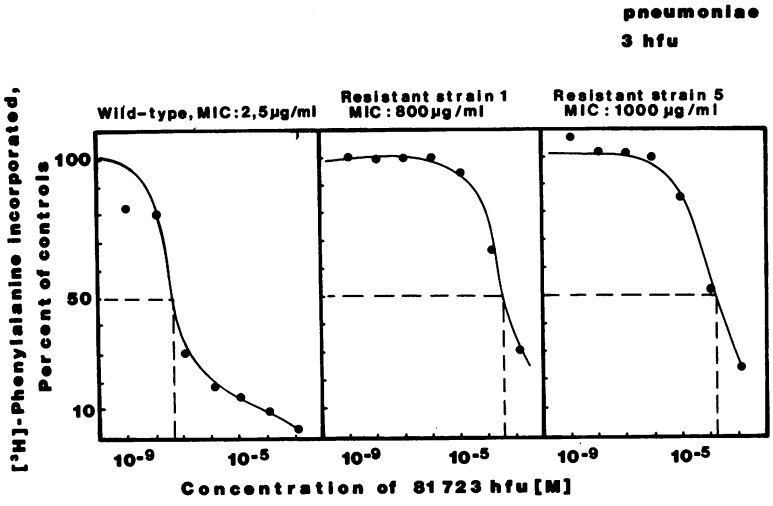

FIG. 3. *Susceptibility of ribosomes from K. pneumoniae wild type (ATCC 10031) and two resistant strains derived from the wild type against 81.723 hfu in a cell-free protein-synthesizing system. Ribosomes from six mutant strains were tested with little difference in the respective results.*

TABLE 7. *Acute toxicity data of 81.723 hfu (milligrams per kilogram body weight)*

Animal species	Sex	Route	LD_{05}	LD_{50}	LD_{95}
Mouse	Female	Peroral	383	841	1,847
Mouse	Female	Subcutaneous	438	521	620
Chicken, 4 weeks	Male	Peroral	1,290	1,860	2,690
Chicken, 4 weeks	Male	Intramuscular	76	270	970
Pigeon	Male	Peroral	484	812	1,365
Pigeon	Female	Peroral	459	917	1,833
Turkey, 3 weeks	Male/female	Peroral	840	1,345	2,154
Turkey, 3 weeks	Male/female	Intramuscular	259	409	645

81.723 hfu was an effective agent in the treatment of a number of experimental infections in mice and rats (Table 8). After subcutaneous application, 81.723 hfu was clearly less effective than tetracycline-hydrochloride and tylosin tartrate in the treatment of infections with β-hemolytic streptococci and *S. aureus*. When given orally, the pleuromutilin derivative was almost as effective as tetracycline against the β-hemolytic *Streptococcus* sepsis, though less effective than the latter compound against the *S. aureus* infection. Mice infected with *Streptococcus aronson* responded slightly better to tylosin tartrate than to 81.723 hfu when both drugs were applied subcutaneously. After oral application, 81.723 hfu was clearly more effective than tylosin tartrate. The chemotherapeutic activity of the new antibiotic against a mycoplasma infection was first tested in the rat polyarthritis. The ED_{50} values obtained in this

model after oral or subcutaneous application of 81.723 hfu were of the same order as the corresponding values for tetracycline. When applied orally, 81.723 hfu was five times as active as tylosin tartrate in the treatment of the mycoplasma-induced rat polyarthritis. It was, however, clearly less effective than tylosin tartrate when both compounds were given subcutaneously. Although these data failed to demonstrate a clear superiority of 81.723 hfu over tylosin tartrate and tetracycline in the mycoplasma arthritis model, they gave an early indication of the possibility that the new antibiotic might have its special merits in the treatment of mycoplasmal rather than bacterial infections. Evidence in support of this proposition will be presented in the accompanying paper (7).

DISCUSSION

Compound 81.723 hfu, like other derivatives of pleuromutilin synthesized in this institute, has certain properties in common with macrolide antibiotics and with lincomycin: all these compounds are active against gram-positive bacteria and mycoplasmas and are only weakly active against gram-negative bacteria. They act bacteriostatically, show greater efficacy at slightly alkaline pH values than at neutral or acidic pH, and interfere with bacterial protein biosynthesis. Differences between the in vitro activities of 81.723 hfu, on the one hand, and of macrolides and lincomycin, on the other hand, appear to be largely quantitative in nature. 81.723 hfu has a 10-fold higher in vitro activity than tylosin tartrate. Whether the efficacy of 81.723 hfu against *Shigella flexneri*, *E. coli*, *K. pneumoniae*, and *P. multocida* which is not found with tylosin tartrate reflects a basic qualitative difference between 81.723 hfu and the macrolides or whether it is only a correlate of the generally higher in vitro activity of 81.723 hfu can only be decided after more extensive comparative studies involving the above-mentioned bacterial species.

As also observed with macrolides, resistance to 81.723 hfu emerges in bacteria in a step wise fashion, but rather rapidly. In mycoplasmas, however, resistance to 81.723 hfu evolves more slowly than in bacteria. Moreover, the levels of resistance to 81.723 hfu attained by two pathogenic mycoplasmas after 20 in vitro passages appear modest compared to the corresponding resistance to tylosin tartrate expressed in these strains after the same number of passages. The complete cross-resistance between 81.723 hfu and erythromycin or lincomycin which was observed in *K. pneumoniae* appears to be in line with the fact that all of these antibiotics are inhibitors of the large (50*S*) subunit of the bacterial ribosome. However, in the two *S. aureus* strains which were investigated, cross-resistance between these antibiotics could not be detected, whereas in *M. gallisepticum* and *M. hyorhinis* cross-resistance between tylosin tartrate and 81.723 hfu was only partial. Therefore, it appears possible that the biochemical mechanisms which produce resistance to 81.723 hfu in staphylococci and mycoplasmas are different from each other as well as from the analogous mechanisms operating in *K. pneumoniae*.

TABLE 8. *Activity of 81.723 hfu, tylosin tartrate, and tetracycline-hydrochloride in experimental infections*

Compound	Model infection	ED_{50} (mg/kg body weight)[a]	
		Subcutaneous	Peroral
81.723 hfu	β-hemolytic *Streptococcus* (mouse)	18.8	43.5
Tylosin tartrate	β-hemolytic *Streptococcus* (mouse)	1.8	62.7
Tetracycline-hydrochloride	β-hemolytic *Streptococcus* (mouse)	2.5	39.0
81.723 hfu	*S. aureus* (mouse)	11.6	48.3
Tylosin tartrate	*S. aureus* (mouse)	1.3	150.0
Tetracycline-hydrochloride	*S. aureus* (mouse)	0.8	7.8
81.723 hfu	*Streptococcus aronson* (mouse)	62.3	85.3
Tylosin tartrate	*Streptococcus aronson* (mouse)	40.8	193.0
Tetracycline-hydrochloride	*Streptococcus aronson* (mouse)		
81.723 hfu	*M. arthritidis* (rat)	26.9	56.5
Tylosin tartrate	*M. arthritidis* (rat)	3.6	281.0
Tetracycline-hydrochloride	*M. arthritidis* (rat)	34.5	31.0

[a] ED_{50}, mean dose curing 50% of the animals from the respective organism.

Data presented in this and in the accompanying paper (7) show that 81.723 hfu is effective in the treatment of model infections with gram-positive bacteria and mycoplasmas. Moreover, as judged by the acute toxicity data in four animal species, the new compound appears to exhibit only low toxicity, especially after oral administration. These findings, as well as the lack of cross-resistance with erythromycin and lincomycin in staphylococci, the slow development of resistance in mycoplasmas, and the fact that mycoplasmas with high levels of resistance to tylosin tartrate appear to lose little of their susceptibility to 81.723 hfu, have made the new antibiotic an interesting candidate for further development. Studies aimed at the complete delineation of this compound's chemotherapeutic potential are now is progress.

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