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Susceptibility of *Pseudomonas* Species to the Novel Antibiotics Mureidomycins

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Strains of *Pseudomonas aeruginosa*, including imipenem- or ofloxacin-resistant clinical isolates, and some other species in the genus *Pseudomonas* were inhibited by novel antibiotics of the mureidomycin (MRD) group. On the other hand, almost all other gram-positive and gram-negative bacteria were resistant to MRDs, though the antibiotics potently inhibited the in vitro peptidoglycan synthesis of *Escherichia coli* and *P. aeruginosa*. All of the strains in the genus *Pseudomonas* that were inhibited by ≤200 μg of MRDs per ml were classified into the rRNA groups I and III, and none of the tested strains of rRNA group I were resistant to MRDs, suggesting that these two groups are closely related to each other evolutionarily. Among group I strains, *P. aeruginosa*, *P. mendocina*, *P. stutzeri*, and *P. alcaligenes* were more susceptible than the others, suggesting a closer relationship among these species.

Mureidomycins (MRDs) A, B, C, and D are novel peptidylnucleoside antibiotics (Fig. 1) produced by *Streptomyces flavidovirens* SANK 60486 (3, 5). Previous studies have shown that they have spheroplast-forming activity (6) and inhibit in vitro peptidoglycan synthesis of *Pseudomonas aeruginosa* (4).

In the course of determining the antimicrobial activity of MRDs, it was found that they were selectively active against *P. aeruginosa* among the gram-positive and gram-negative bacteria tested. Since no antibiotic has been reported to have such a selective antipseudomonal activity, we tested the more precise antibacterial activity of MRDs against many representatives of gram-positive and gram-negative bacteria as well as various species of the genus *Pseudomonas*, including fresh clinical isolates. Since a classification of *Pseudomonas* species into five groups is proposed by Palleroni on the basis of rRNA similarity (11), we used the type strains of the *Pseudomonas* species included in Palleroni's five groups. In this paper we report that MRDs are active against clinical isolates of *P. aeruginosa*, including β-lactam- or quinolone-resistant strains, and that the antipseudomonal activity of MRDs is restricted to all species of *Pseudomonas* group I and certain species of *Pseudomonas* group III.

MATERIALS AND METHODS

Organisms. All strains except fresh clinical isolates were stock cultures of Tsukuba Research Laboratory, Sankyo Co., Ltd. Clinical isolates were obtained from various hospitals in Japan between 1988 and 1990.

Susceptibility study. An overnight culture of each organism in nutrient broth (Eiken) was diluted to give an inoculum of 10^6 CFU/ml, and a 10-μl aliquot was spotted onto a Mueller-Hinton agar plate containing twofold dilutions of the antibiotic. Plates were incubated for 24 h at 37°C, and the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of bacteria. MICs of cefsulodin and ceftazidime were determined in parallel by the same method.

In vitro peptidoglycan synthesis. The assay of in vitro peptidoglycan synthesis in ether-treated cells was performed as described previously (4). Ether-treated cells were prepared according to the method of Vosberg and Hoffmann-Berling (14), and the incorporation of UDP-U-^{14}C-*N*-acetylglucosamine into cold 5% trichloroacetic acid-insoluble fractions was measured.

Chemicals. MRDs A, B, C, and D were prepared from the fermentation broth of *S. flavidovirens* SANK 60486 as described previously (3). UDP-U-^{14}C-*N*-acetylglucosamine was purchased from Amersham Japan. Cefsulodin and ceftazidime were obtained commercially.

RESULTS

The antimicrobial spectrum of MRDs. The antimicrobial activities of MRDs A, B, C, and D against representative gram-positive and gram-negative bacteria are shown in Table 1. Generally, MRDs were not active against gram-positive organisms, with MICs equalling 200 μg/ml or more. Most of the gram-negative organisms were resistant, requiring MICs greater than 200 μg/ml. By contrast, two *P. aeruginosa* strains were inhibited by MRDs, requiring MICs of ≤6.25 μg/ml.

Antimicrobial activities of MRDs against *P. aeruginosa*. Table 2 summarizes the activities of MRDs against various strains of *P. aeruginosa*, including fresh clinical isolates. Except for one clinical isolate, *P. aeruginosa* 2093, all strains tested were inhibited by MRDs at 0.05 to 12.5 μg/ml. Although *P. aeruginosa* 2093 was less susceptible, MRD C inhibited its growth at 25 μg/ml. These potencies of MRDs against *P. aeruginosa* were comparable to those of β-lactams such as cefsulodin and ceftazidime.

The recent emergence in hospitals of antibiotic-resistant bacteria resistant not only to β-lactams such as ceftazidime and imipenem but also to the new quinolones is becoming a very severe problem. Clinical isolates of imipenem- or ofloxacin-resistant strains were also inhibited by MRDs A and C. The MIC ranges of MRDs A and C for nine ofloxacin-resistant *P. aeruginosa* strains (MICs ≥100 μg/ml) were 3.13 to 25 μg/ml for both compounds, and those for six imipenem-

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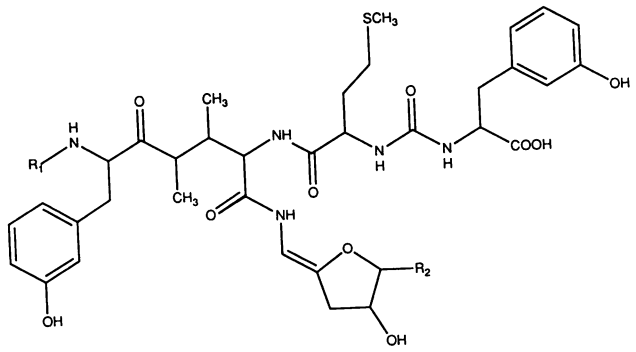

	R1	R2
MRD A	H	Uracil
MRD B	H	Dihydrouracil
MRD C	Glycine	Uracil
MRD D	Glycine	Dihydrouracil

FIG. 1. Structures of MRDs A, B, C, and D.

resistant strains (MICs of 25 or 50 μg/ml) were 3.13 to 25 μg/ml and 0.78 to 25 μg/ml, respectively.

Activities of MRDs against various *Pseudomonas* species. Table 3 shows MICs against *Pseudomonas* strains of the various species belonging to the five rRNA groups of Palleroni (11). *P. acidovorans*, *P. testosteroni*, and *P. maltophilia* had been included in the genus *Pseudomonas* and classified by Palleroni into groups III, III, and V, respectively. However, they were recently transferred to the genera *Comamonas* (13), *Comamonas* (13), and *Xanthomonas* (12), respectively. As shown in Table 3, organisms classified into group I were inhibited by MRDs A and C. Among them, *P. aeruginosa*, *P. mendocina*, *P. stutzeri*, and *P. alcaligenes* required MIC ranges of 0.8 to 3.13 μg/ml. Other species in group I, such as *P. fluorescens*, *P. putida*, *P. aureofaciens*, and *P. syringae*, were less susceptible to MRDs A and C. Among species in group III, *P. acidovorans*, *P. palleronii*, *P. delafieldii*, *P. flava*, and *P. pseudoflava* required levels of MRDs A and C up to 200 μg/ml for inhibition, while one *P. testosteroni* isolate was not inhibited. All other pseudomonads, including *P. cepacia* in group II, *P. diminuta* and *P. vesicularis* in group IV, and *P. maltophilia* in group V, were resistant to MRDs.

Additionally, the MICs for the *Pseudomonas* strains that are included in section V in *Bergey's Manual of Systematic Bacteriology* as unclassified pseudomonads (11) are shown in Table 3. Among them, *P. straminea*, *P. mucidorens*, *P. nitroreducens*, *P. fluva*, *P. paucimobilis*, *P. taetrolens*, and *P. azotoformans* were inhibited, while *P. pictorum* and *P. glumae* were not.

Effect of MRDs on in vitro peptidoglycan synthesis in *Escherichia coli*. In a previous study it was demonstrated that MRDs inhibited the growth of *P. aeruginosa* by interfering with peptidoglycan synthesis (4). In vitro peptidoglycan synthesis in ether-permeabilized *P. aeruginosa* was completely inhibited by MRDs at a very low concentration. Therefore, the effect of MRD A on in vitro peptidoglycan synthesis was examined with ether-treated cells of *E. coli* NIHJ. As shown in Table 4, *E. coli* NIHJ was extremely resistant to MRDs, but in vitro peptidoglycan synthesis was inhibited by MRD A to the same extent as *P. aeruginosa* at drug concentrations of 1 and 10 μg/ml.

DISCUSSION

P. aeruginosa often causes opportunistic infections in immunocompromised patients, and one of the problems associated with achieving successful therapy is the intrinsic

TABLE 1. Antimicrobial spectra of MRDs

Organism	MIC (μg/ml)[a] of:			
	MRD A	MRD B	MRD C	MRD D
Staphylococcus aureus FDA 209P	200	>200	>200	>200
S. aureus 56 SANK 70175	200	>200	200	200
S. aureus 337 SANK 71075	>200	>200	>200	>200
S. aureus 108-1 (MRSA[b])	>200	>200	>200	>200
S. aureus 123-1 (MRSA)	200	>200	>200	>200
S. aureus 2-1 (MRSA)	200	>200	>200	>200
S. aureus Smith SANK 71182	200	200	200	200
Staphylococcus epidermidis SANK 71575	>200	>200	>200	>200
Enterococcus faecalis SANK 71478	>200	>200	>200	>200
Bacillus subtilis ATCC 6633	>200	>200	>200	>200
E. coli NIHJ JC-2	>200	>200	>200	>200
E. coli 97 SANK 71775	>200	>200	>200	>200
E. coli 665 SANK 72875	>200	>200	>200	>200
Enterobacter cloacae IAM 1134	>200	>200	>200	>200
Proteus mirabilis RMS 203	>200	>200	>200	>200
P. mirabilis SANK 71873 (Hep^-)	>200	>200	>200	>200
Proteus vulgaris SANK 73266	>200	ND	ND	ND
Providencia rettgeri SANK 73775	>200	ND	ND	ND
Klebsiella pneumoniae PCI 806	>200	ND	>200	ND
K. pneumoniae PCI 846	>200	>200	>200	>200
Serratia marcescens SANK 73060	>200	>200	>200	>200
P. aeruginosa NCTC 10490	0.2	0.2	0.2	0.78
P. aeruginosa ATCC 13383	1.56	3.13	1.56	6.25

[a] MICs were determined as described in Materials and Methods. ND, not determined.
[b] MRSA, methicillin-resistant *S. aureus*.

TABLE 2. Antimicrobial activities of MRDs, cefsulodin, and ceftazidime against *P. aeruginosa*

P. aeruginosa strain	MIC (μg/ml)[a] of:					
	MRD A	MRD B	MRD C	MRD D	CFS	CAZ
NCTC 10490	0.2	0.2	0.2	0.78	0.4	0.1
ATCC 13388	1.56	3.13	1.56	6.25	0.1	<0.05
NRRL B-100	3.13	6.25	3.13	6.25	1.56	0.4
SC 8753	3.13	3.13	1.56	6.25	6.25	1.56
SANK 73575	3.13	6.25	1.56	6.25	6.25	1.56
SANK 75775	1.56	3.13	1.56	6.25	6.25	1.56
SANK 75175	3.13	12.5	3.13	12.5	12.5	6.25
SANK 70479	1.56	1.56	1.56	6.25	100	1.56
SANK 70579	0.05	0.05	0.05	0.1	3.13	0.78
433[b]	0.78	3.13	1.56	6.25	3.13	0.78
638[b]	3.13	6.25	3.13	12.5	3.13	1.56
2093[b]	50	50	25	100	3.13	0.78
4-1068[b]	3.13	6.25	3.13	12.5	6.25	1.56

[a] MICs were determined by agar dilution. CFS, cefsulodin; CAZ, ceftazidime.
[b] Clinical isolates.

TABLE 3. Activities of MRDs against various *Pseudomonas* species classified on the basis of rRNA similarity

Pseudomonas species[a]	MIC (μg/ml)		rRNA group[b]
	MRD A	MRD C	
P. aeruginosa ATCC 10145^T	6.25	3.13	I
P. aeruginosa ATCC 13388	1.56	1.56	I
P. mendocina ATCC 25411^T	3.13	3.13	I
P. stutzeri ATCC 17588^T	3.13	3.13	I
P. alcaligenes ATCC 14909	1.56	0.78	I
P. fluorescens IAM 1178	12.5	12.5	I
P. putida ATCC 12633^T	25	12.5	I
P. putida IAM 1002	25	12.5	I
P. putida IAM 1050	25	25	I
P. aureofaciens ATCC 13985^T	100	25	I
P. aureofaciens NRRL B-1543	25	25	I
P. syringae SANK 70770	25	50	I
P. cepacia ATCC 25416^T	>200	>200	II
P. cepacia ATCC 17759	>200	>200	II
P. acidovorans ATCC 15668^T	100	50	III
P. testosteroni ATCC 11996^T	>200	>200	III
P. palleronii ATCC 17724^T	100	100	III
P. delafieldii DSM 50263	200	200	III
P. flava DSM 619^T	50	12.5	III
P. pseudoflava DSM 1034^T	50	12.5	III
P. diminuta ATCC 11568^T	>200	>200	IV
P. vesicularis ATCC 11426	>200	>200	IV
P. maltophilia ATCC 13637^T	>200	>200	V
P. maltophilia SANK 75285	>200	>200	V
P. straminea IAM 1598^T	1.56	1.56	Unclassified
P. mucidolens ATCC 4685^T	3.13	3.13	Unclassified
P. fluva IAM 1529^T	6.25	6.25	Unclassified
P. nitroreducens IAM 1439^T	12.5	6.25	Unclassified
P. taetrolens ATCC 4683^T	25	6.25	Unclassified
P. azotoformans IAM 1603^T	100	100	Unclassified
P. paucimobolis ATCC 29837^T	25	12.5	Unclassified
P. pictorum NCBI 9152^T	>200	>200	Unclassified
P. glumae SANK 79585	>200	>200	Unclassified

[a] T, type strain.
[b] rRNA similarity group described by Palleroni (11).

resistance of the organism to many antibiotics, resulting from low outer membrane permeability (9). MRDs are unique in that they are selectively active against several species of the genus *Pseudomonas*, including *P. aeruginosa*. Therapy against *P. aeruginosa* infection has been improved by introduction of imipenem and new quinolones, but resistance to these and other drugs is becoming a problem (2, 7). For this reason, it is interesting that MRDs were active against the imipenem- or quinolone-resistant clinical isolates included in this study. In a previous paper, we reported that MRDs inhibit the peptidoglycan synthesis of *P. aeruginosa* through the phospho-MurNAc-pentapeptide translocase located at the inner surface of the cytoplasmic membrane (4). As shown in this paper, in vitro peptidoglycan synthesis in *E. coli* was inhibited to an extent similar to that in *P. aeruginosa*, indicating that the target enzyme of the resistant bacteria is still sensitive to MRDs. We also tested for the inactivation of MRDs by intact cells or cell lysates of MRD-resistant bacteria such as *E. coli* or a laboratory-derived mutant of *P. aeruginosa* but could not detect any inactivating activity (data not shown). These results suggest that resistance to MRDs may be due to a permeability barrier against these agents. The lipid bilayer of the cytoplasmic membrane is thought to be a common barrier in gram-positive and gram-negative bacteria against MRDs because of their hydrophilicity. In addition, gram-negative bacteria have an outer membrane that allows the penetration of only small hydrophilic molecules. Permeability of large hydrophilic solutes through the outer membrane of *P. aeruginosa* is especially low (9), and no antibiotics as large as MRDs, whose molecular masses range from 840 to 899 Da, have been demonstrated to effectively penetrate the outer membrane of *P. aeruginosa*. From these observations it could be hypothesized that *P. aeruginosa* and related species might have a specific channel or channels in the outer and/or inner membrane that allow the effective permeation of MRDs.

It should be noted that all *Pseudomonas* strains were not susceptible to MRDs. Within the tested strains organisms which were inhibited by MRDs were all included in Palleroni's rRNA groups I and III; moreover, all strains belonging to group I were inhibited by MRDs. Some of the strains in Table 3, which are not classified by Palleroni, were classified by Oyaizu and Komagata on the basis of their cellular fatty acid compositions and quinone systems (10). Oyaizu and Komagata reported that *P. straminea*, *P. nitroreducens*, *P. fluva*, *P. azotoformans*, *P. taetrolens*, and *P. mucidolens* should be included in group I and that *P. pictorum* should be included in group V. Again, all strains in group I were inhibited by MRDs. It is therefore suggested that the growth inhibition by MRDs is a property against all pseudomonads in group I and some in group III, such as *P. acidovarans*, *P. palleronii*, *P. delafieldii*, *P. flava*, and *P. pseudoflava*. These

TABLE 4. Effect of MRD A on in vitro peptidoglycan synthesis in *E. coli* and *P. aeruginosa*

MRD A (μg/ml)[a]	Inhibition (%)[b] of:	
	E. coli NIHJ	*P. aeruginosa* SANK 75775
10	100	100
1	100	94
0.1	91	30

[a] MICs of MRD A for *E. coli* and *P. aeruginosa* SANK 75775 were >200 and 1.56 μg/ml, respectively.
[b] In vitro peptidoglycan synthesis in ether-treated bacteria was measured as described previously (4).

species seem to be more closely related to group I genealogically than to the other groups.

It is also interesting that *P. mendocina*, *P. stutzeri*, and *P. alcaligenes* were as susceptible as *P. aeruginosa*. Although phenotypic characterization suggested a distinct cluster of these species for the nonfluorescent species (1), they were shown to have significant similarity to *P. aeruginosa* in DNA-DNA hybridization experiments (8). Susceptibility to MRDs seems to be further evidence for the close relationship between these species and *P. aeruginosa*.

The mechanism which affords certain *Pseudomonas* species the property of MRD susceptibility is very interesting. We hypothesize that this ability is associated with the uptake of some nutrient from the environment that is necessary for cell survival under certain growth conditions. From the results given in this paper, it is hypothesized that they would have obtained this MRD permeation system during evolutionary divergence to group I and III, since it seems unlikely that such a rare mutation could occur twice.

Classification of the genus *Pseudomonas* seems somewhat uncertain. The observed range of antipseudomonal activity of MRDs may reflect the incomplete classification of this genus. Antimicrobial susceptibility to the MRDs may be a useful marker by which to aid classification of the species in this genus.

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REFERENCES

1. **Gavini, F., B. Holmes, D. Izard, A. Beji, A. Bernigaud, and E. Jakubczak.** 1989. Numerical taxonomy of *Pseudomonas alcaligenes*, *P. pseudoalcaligenes*, *P. mendocina*, *P. stuzeri*, and related bacteria. Int. J. Syst. Bacteriol. **39:**135–144.
2. **Giamarellou, H., and N. Galankis.** 1987. Use of intravenous ciprofloxacin in difficult to treat infection. Am. J. Med. **82**(Suppl. 4A):346–351.
3. **Inukai, M., F. Isono, S. Takahashi, R. Enokita, Y. Sakaida, and T. Haneishi.** 1989. Mureidomycins A–D, novel peptidylnucleoside antibiotics with spheroplast forming activity. I. Taxonomy, fermentation, isolation and physico-chemical properties. J. Antibiot. **42:**662–666.
4. **Isono, F., and M. Inukai.** 1991. Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. Antimicrob. Agents Chemother. **35:**234–236.
5. **Isono, F., M. Inukai, S. Takahashi, T. Haneishi, T. Kinoshita, and H. Kuwano.** 1989. Mureidomycins A–D, novel peptidylnucleoside antibiotics with spheroplast forming activity. II. Structural elucidation. J. Antibiot. **42:**667–673.
6. **Isono, F., T. Katayama, M. Inukai, and T. Haneishi.** 1989. Mureidomycins A–D, novel peptidylnucleoside antibiotics with spheroplast forming activity. III. Biological properties. J. Antibiot. **42:**674–679.
7. **Johnson, J., and N. J. Palleroni.** 1989. Deoxyribonucleic acid similarities among *Pseudomonas* species. Int. J. Syst. Bacteriol. **39:**230–235.
8. **Lynch, M. J., G. L. Drusano, and H. L. T. Mobley.** 1987. Emergence of resistance to imipenem in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**1892–1896.
9. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:** 1831–1836.
10. **Oyaizu, H., and K. Komagata.** 1983. Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference of 3-hydroxy fatty acid. J. Gen. Appl. Microbiol. **29:**17–40.
11. **Palleroni, N. J.** 1984. Genus I. *Pseudomonas* Migula 1984, 237^{AL}, p. 141–199. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
12. **Swings, J., P. De Vos, M. Van den Mooter, and J. De Ley.** 1983. Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. Int. J. Syst. Bacteriol. **33:**409–413.
13. **Tamaoka, J., D.-M. Ha, K. Komagata.** 1987. Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. Int. J. Syst. Bacteriol. **37:**52–59.
14. **Vosberg, H. P., and H. Hoffmann-Berling.** 1971. DNA synthesis in nucleotide permeable *Escherichia coli* cells. I. Preparation and properties of ether-treated cells. J. Mol. Biol. **58:**739–753.