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Enzymatic Inactivation of a New Aminoglycoside Antibiotic, Sisomicin, by Resistant Strains of *Pseudomonas aeruginosa*

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The antibacterial activity of sisomicin (SS), a new aminoglycoside antibiotic active toward clinical isolates of *Pseudomonas aeruginosa*, was determined and compared with that of the gentamicin C complex. Both drugs were effective against these strains and showed almost the same antibacterial activity. A few strains were found to be resistant to SS. The antibiotic was inactivated by a cell-free extract from the SS-resistant strains due to acetylation of the drug. Comparative studies of the inactivation of the drugs which lack a 6'-amino group in the amino sugar linked to 2-deoxystreptamine strongly suggested that SS inactivation was due to acetylation of the 6'-amino group of the 4',5'-didehydropurpurosamine moiety.

Sisomicin (SS) was isolated from the fermentation broth of *Micromonospora inyoensis* (9) and has proved effective against *Pseudomonas aeruginosa* (8).

This paper details the antibacterial activity of SS toward *P. aeruginosa* and the mechanism of SS inactivation by a cell-free extract from SS-resistant strains.

MATERIALS AND METHODS

Bacterial strains. Four hundred and fifty-seven strains of *P. aeruginosa* isolated from clinical sources were used for the determination of drug resistance. *Bacillus subtilis* PCI-219 was used as a test strain for microbiological assay. *P. aeruginosa* GN150, sensitive to both SS and gentamicin (GM), was also used as a reference strain.

Chemicals. SS and each component of the GM-C complex were obtained from M. J. Weinstein, the Shering Co., Ltd. Adenosine 5'-triphosphate, ^{14}C-labeled sodium acetate (CH_3 $^{14}COONa$; specific activity, 48.0 mCi/mmol), 2,5-diphenyloxazole, and dimethyl-1,4-bis-(5-phenyloxazolyl)-benzene (Daiichi Kagaku Co.), and coenzyme A (CoA) and acetyl-CoA (Sigma Co.) were all commercial products. Phosphocellulose paper (Whatman P 81) was commercially purchased.

Media. Nutrient broth and nutrient agar were used for bacterial growth.

Determination of antibacterial activity. An overnight culture of the bacterial strain was spotted with a capillary onto nutrient agar plates containing serial twofold dilutions of drug. After incubation for 24 h, minimal inhibitory concentration (MIC) of bacterial growth was scored.

Preparation of cell-free extract. Bacterial cells in exponentially growing culture were harvested by centrifugation at 5,000 × *g* for 15 min and washed with TMK solution [0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.8, 0.06 M KCl, 0.01 M magnesium acetate, and 6 mM 2-mercaptoethanol]. Washed cells obtained from a 1,000-ml culture were suspended in 3.0 ml of TMK solution and disintegrated by sonic oscillation (Ohtake Sonicator, Tokyo; 20 kc) for 5 min. The supernatant fluid obtained by centrifugation at 30,000 × *g* for 2 h was further centrifuged at 105,000 × *g* for 2 h. The final supernatant fluid thus obtained was used as a cell-free extract.

For study of the incorporation of ^{14}C-labeled acetate into SS, a cell-free extract was prepared by using modified TMK solution which consisted of 0.1 M Tris-hydrochloride buffer, pH 7.8, 0.06 M KCl, 0.01 M $MgCl_2$, and 6 mM 2-mercaptoethanol. Protein determinations were carried out by Lowry's method (4).

Inactivation of antibiotic by cell-free extracts. Crude enzyme solution (0.3 ml), 0.1 ml of 40 mM adenosine 5'-triphosphate, 0.1 ml of 2 mM CoA, 0.1 ml of 1 mM antibiotic, and 0.4 ml of TMK solution were mixed and incubated at 37 C for 2 h. After heating at 100 C for 3 min, the residual activity of SS in the reaction mixture was determined by using *B. subtilis* PCI 219 as a test organism.

Incorporation of ^{14}C-labeled acetate into SS. The reaction mixture contained 5 μliters of crude enzyme solution which was adjusted to 30 mg of protein concentration per ml, 5 μliters of ^{14}C-labeled acetate (1 μCi), 5 μliters of 2 mM CoA, 5 μliters of 1 mM antibiotic, 5 μliters of 40 mM adenosine 5'-triphosphate, and 25 μliters of modified TMK solution at pH 7.8. The reaction mixture was incubated at 37 C for 1 h, and 10 μliters was pipetted onto a 0.75-cm^2 square of phosphocellulose paper. The square was then immersed in hot distilled water (70 to 80 C) for 2 min to stop the reaction, washed five times with 20 ml of hot distilled water, and dried, and the radioactivity

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was counted in an Aloka liquid scintillation counter (TRM-502) using a toluene-based fluid [2,5-diphenyloxazole, 5 g, and dimethyl-1,4-bis-(5-phenyloxazolyl)-benzene, 0.3 g, in 1 liter of toluene]. The ^{14}C-labeled sodium acetate nonspecifically bound to the paper was determined as a control in the reaction mixture without either enzyme or antibiotic.

RESULTS

Antibacterial activity of SS and GM toward P. aeruginosa. The MIC distribution pattern was examined using 457 strains of *P. aeruginosa*. As shown in Fig. 1, the MIC distribution patterns of both antibiotics exhibited a single peak, with a maximum at 6.25 μg of each drug per ml. This indicates that, in the MIC test, growth at 25 μg/ml is a criterion of resistance to these antibiotics.

Inactivation of SS by cell-free extracts from resistant strains. Inactivation of SS by cell-free extracts from SS-resistant strains was examined (Table 1). Complete inactivation of SS was observed in four SS-resistant strains, which were resistant to 25 μg or more of SS per ml. No significant inactivation of SS was observed in the remaining strains in which the MIC of SS was 25 μg/ml or less.

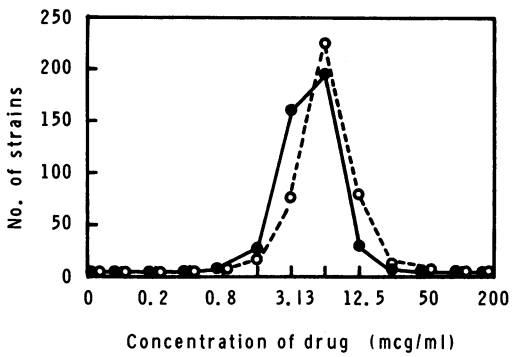

FIG. 1. *Antibacterial activity of SS and GM against 457 strains of P. aeruginosa. Symbols: ●, SS; ○, GM.*

TABLE 1. *SS resistance in strains of P. aeruginosa and inactivation of the drug*

Strains	MIC (μg/ml)	Inactivation (%)
GN269	200	100
GN315	200	100
GN362	100	100
GN314	50	100
GN1590	25	0
GN2790	25	0
GN2971	25	9
GN2972	25	13
GN2977	25	9
GN150	3.13	11

Optimal conditions for the inactivating reaction. The optimal pH for the SS inactivation was about 7.5, and no significant difference was seen between pH 7.0 and 8.0. However, the inactivating activity was decreased at pH values below 6.5 or over 8.5 (Fig. 2). The optimal temperature for the SS inactivation was 37 to 40 C, and the activity was greatly decreased at temperatures below 30 or over 50 C (Fig. 3).

Mechanism of SS inactivation. Cell-free extracts from SS-resistant strains were used to elucidate the mechanism of SS inactivation. As shown in Table 2, SS was inactivated in the complete system, indicating that SS inactivation is due to acetylation of the drug.

SS-resistant strains GN269, GN314, GN315, and GN362 could inactivate the drug in the presence of acetyl CoA. The incorporation of ^{14}C-labeled acetate into SS was demonstrated by cell-free extracts from SS-resistant strains parallel with the SS inactivation.

To learn more about the mechanism of SS inactivation, GM-C_1 and -C_{1a}, and kanamycins (KM)-A and -C were used as substrates, in addition to SS. As shown in Table 4, the cell-free extracts from SS-resistant strains

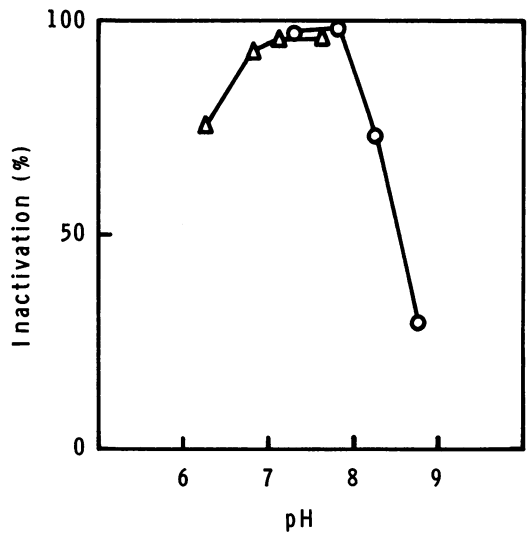

FIG. 2. *Effect of pH for SS inactivation by cell-free extract of P. aeruginosa GN269. Crude enzyme solution prepared by the method described in Materials and Methods was dialyzed against 0.1 M Tris-hydrochloride buffer or Sörensen phosphate buffer containing 0.06 M KCl, 0.01 M $Mg(CH_3COO)_2$, and 6 mM 2-mercaptoethanol at various pH values, as indicated in the figure. After incubation for 2 h at 37 C, residual potency of SS in the reaction mixture was determined by bioassay. Phosphate buffer, Δ; Tris-hydrochloride buffer, ○.*

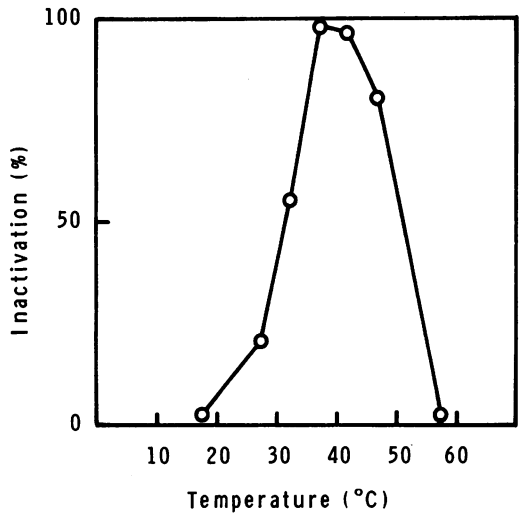

FIG. 3. *Effect of temperature for SS inactivation by cell-free extract of P. aeruginosa GN269. After 2 h of incubation of the reaction mixture (see Materials and Methods) at various temperatures as indicated in the figure, the residual potency of SS in each reaction mixture was determined by bioassay.*

TABLE 2. *Requirements for the inactivation of SS by cell-free extracts from P. aeruginosa GN269*

Reaction mixture	Inactivation (%)
Complete system[a]	100
- ATP	4
- CoA	0
- $Mg(CH_3COO)_2$	2
+ acetyl CoA (2 mM)	100
- ATP - CoA	
Control[b]	0

[a] For details, see Materials and Methods. ATP, adenosine 5'-triphosphate.

[b] In control, cell-free extract heated at 100 C for 3 min was used.

could inactivate SS, GM-C_{1a} and KM-A, but did not in activate either GM-C_1 or KM-C. Similarly, the incorporation of ^{14}C-labeled acetate into drugs could not be demonstrated when either GM-C_1 or KM-C was used as substrate. These results strongly suggest that SS inactivation by resistant strains was due to acetylation of the 6'-amino group in the 4',5'-didehydropurpurosamine moiety.

DISCUSSION

SS and GM-C_{1a} are very closely related compounds, the only difference being the presence of the 4',5'-dihydropurporosamine ring in the former and a purpurosamine ring in the latter. The MIC distribution patterns of SS and GM in *P. aeruginosa* were almost the same.

TABLE 3. *Incorporation of $CH_3{}^{14}COONa$ into SS and inactivation of the drug by cell-free extracts from SS-resistant strains of P. aeruginosa*

Cell-free extract from	Incorporation of ^{14}C-labeled acetate[a]	Inactivation (%)[a]
GN269	3,542	91
GN315	2,570	78
GN362	2,445	81
GN314	2,145	72
GN1590	80	0
GN2790	77	9
GN2971	46	8
GN2972	100	11
GN2977	36	0
GN150	22	0

[a] For details, see Materials and Methods. Counts were presented by counts per minute of ^{14}C-labeled acetate onto phosphocellulose paper. A background incorporation of radioactivity from a control, described in Materials and Methods, was subtracted to obtain the values listed above.

It was reported by H. Umezawa et al. (7) that KM-A and -B were inactivated by a KM-resistant strain of *Escherichia coli* carrying NR79 factor, and by *P. aeruginosa*, by means of acetylation of the 6'-amino group of the 6-aminoglucose moiety.

Benveniste and Davis (1) reported that GM-C_{1a} and -C_2, as well as the kanamycins, were inactivated by a cell-free extract from *E. coli* NR79 due to acetylation of the 6'-amino group of the purpurosamine ring, but there was no acetylation of GM-C_1.

Mitsuhashi et al. (5, 6) reported that GM-C_1, -C_{1a}, and -C_2 were inactivated by *P. aeruginosa* 99 and cape 18, and the mechanism of inactivation was found to be due to acetylation of the 3-amino group of 2-deoxystreptamine (2, 3).

It was reported in the previous paper (6) that SS was also inactivated by SS-resistant strains of *P. aeruginosa*, due to acetylation. The results described in the present communication indicate that the SS-inactivating enzyme could acetylate SS, GM-C_{1a}, and KM-A, but could not inactivate GM-C_1 and KM-C, which lack a 6'-amino group in the amino sugar linked to the 2-deoxystreptamine moiety. The data strongly suggest that SS inactivation was due to acetylation of the 6'-amino group of the 4',5'-dideoxypurpurosamine ring. Detailed studies of the inactivated position of SS will be described elsewhere.

TABLE 4. *Incorporation of labeled acetate into drugs and their inactivation*

Cell-free extract from	Incorporation of ^{14}C-labeled acetate into				
	SS	GM-C_{1a}	GM-C_1	KM-A	KM-C
GN269	3,030 (83)[a]	3,100 (85)	64 (0)	2,630 (81)	92 (0)
GN315	2,878 (70)	2,874 (75)	32 (16)	2,508 (94)	58 (15)
GN362	2,844 (74)	3,276 (90)	72 (0)	3,666 (100)	83 (15)
GN314	2,430 (73)	2,890 (80)	70 (0)	3,400 (100)	90 (16)
GN150	82 (8)	45 (0)	36 (0)	36 (11)	23 (0)

[a] Numbers in parentheses indicate percentage of inactivation of drugs. Residual potency of each drug was determined by bioassay after incubation. For details, see Materials and Methods.

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LITERATURE CITED

1. Benveniste, R., and J. Davies. 1971. Enzymatic acetylation of aminoglycosidics by *Escherichia coli* carrying an R factor. Biochemistry **10**:1787-1796.
2. Brzenzinska, M., R. Benveniste, J. Davies, P. J. L. Daniels, and J. Weinstein. 1972. Gentamicin resistance in strains of *Pseudomonas aeruginosa* mediated by enzymatic N-acetylation of the deoxystreptamine moiety. Biochemistry **11**:761-766.
3. Kobayashi, F., M. Yamaguchi, J. Eda, M. Hiramatsu, and S. Mitsuhashi. 1972. Gentamicin C acetylating enzyme from resistant strains of *Pseudomonas aeruginosa* and the structure of the acetylated product. Gunma Rep. Med. Sci. **5**:291-301.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
5. Mitsuhashi, S., F. Kobayashi, and M. Yamaguchi. 1971. Enzymatic inactivation of gentamicin C components by cell-free extract from *Pseudomonas aeruginosa*. J. Antibiot. **24**:400-401.
6. Mitsuhashi, S., F. Kobayashi, M. Yamaguchi, K. O'Hara, and M. Kono. 1971. Enzymatic inactivation of aminoglycoside antibiotics by resistant strains of bacteria, p. 561-564. Proc. 7th Int. Congr. of Chemother., Vienna.
7. Umezawa, H., M. Okanishi, R. Utahara, K. Maeda, and S. Kondo. 1967. Isolation and structure of kanamycin inactivated by a cell free system of kanamycin-resistant *E. coli*. J. Antibiot. **20**:136-141.
8. Waitz, J. A., E. L. Moss, J. M. Oden, and M. J. Weinstein. 1970. Antibiotic 6610. III. Biological studies with antibiotic 6610, a new broadspectrum aminoglycoside antibiotic. J. Antibiot. **23**:559-565.
9. Weinstein, M. J., J. A. Marquez, R. Testa, G. H. Wagman, and J. A. Waitz. 1970. Antibiotic 6610. A new Micromonospora-produced aminoglycoside antibiotic. J. Antibiot. **23**:551-554.