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Enzymatic Modification of Aminoglycoside Antibiotics: a New 3-*N*-Acetylating Enzyme from a *Pseudomonas aeruginosa* Isolate

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A new 3-*N*-aminoglycoside acetyltransferase is described, which possesses a wider substrate range than any such enzyme so far discovered in clinical isolates of antibiotic-resistant bacteria.

Since the introduction of the 3'-deoxyaminoglycoside antibiotics (gentamicin, tobramycin, and dideoxykanamycin), a number of new aminoglycoside-modifying enzymes have been detected in resistant strains of bacteria. Among these, 3-*N*-acetylation appears to be a widespread type of modification, having been detected in resistant strains isolated in South Africa, Europe, Japan, and North America. The majority of these reports have concerned clinical isolates of *Pseudomonas aeruginosa*, but *Escherichia coli* strains carrying the same resistance mechanism, encoded by a resistance plasmid, have also been isolated. Le Goffic and co-workers (7) recently described a 3-*N*-acetyltransferase that appears to differ in substrate range and certain physical properties from the enzyme first reported by Brzezinska et al. (1). The two enzymes differ slightly in their ability to acetylate the antibiotic tobramycin; however, neither of the enzymes is capable of catalyzing the acetylation of neomycin.

We now wish to report the characterization of a distinct form of 3-*N*-acetyltransferase that has a very broad substrate range, including neomycin.

(A preliminary report of these data was presented at the Second International Symposium on Antibiotic Resistance: Drug Inactivating Enzymes and Other Problems of Resistant Bacteria, Castle of Smolenice, Czechoslovakia, 1974.)

METHODS AND MATERIALS

Bacterial strains. *P. aeruginosa* Travers (henceforth referred to as *P. aeruginosa* PST) was kindly provided by J. A. Waitz. *E. coli* carrying R135 was the gift of Y. A. Chabbert; *P. aeruginosa* 130 and 209 have been described previously (1).

Preparation of inactivating enzyme and enzymatic assays and purification of acetylated products. These preparations and purifications are described in the accompanying paper (4). The enzyme was purified roughly 10-fold.

General analytical methods. Proton magnetic resonance (PMR) spectra were measured on a Varian Associates XL-100 spectrometer in D_2O solutions. Chemical shifts (δ) are reported in parts per million from external sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Low-resolution mass spectra were obtained on an Atlas CH-5 mass spectrometer with a heated direct inlet system. High-resolution mass spectra were obtained using a CEC 21-110B spectrometer. Thin-layer chromatography (TLC) was performed on Analtech silica gel plates.

Degradation of *N*-acetylsisomicin from *E. coli* R135/C600. 3-*N*-acetylsisomicin (32 mg) in ethanol (2 ml) was treated with *p*-methoxybenzaldehyde (820 mg), and the solution was allowed to stand for 16 h at room temperature. The solution was evaporated in vacuo, and the residue was taken up in chloroform (2 ml) and added dropwise to 10 ml of hexane. The precipitate was filtered off and dried, affording tetrabenzylidine-3-*N*-acetylsisomicin (Fig. 1, 2) (62 mg).

The benzylidine derivative (62 mg) in ethanol (2 ml) was treated with sodium borohydride (45 mg). After standing for 16 h, the solution was evaporated to dryness and redissolved in 6 N hydrochloric acid. The solution was heated under reflux for 2 h and then evaporated in vacuo. The residue was dissolved in water and neutralized with Amberlite IR 401S resin ($^-$OH form), and the solution was lyophilized. The product was chromatographed over silica gel (10 g), using the lower phase of a chloroform-methanol-15% ammonium hydroxide (1:1:1) solvent system as eluant. Appropriate fractions, as determined by TLC, were combined to give (3) (3.5 mg, 19%).

RESULTS

Resistance characteristics of *P. aeruginosa* PST. The spectrum of resistance of the clinical isolate *P. aeruginosa* PST is shown in Table 1 compared with strains *P. aeruginosa* 209 and *E. coli* carrying resistance plasmid R135. The

latter strains are resistant by virtue of the presence of 3-*N*-acetyltransferase AAC(3)-I. It is clear that the resistance phenotype of *P. aeruginosa* PST is quite different from *P. aeruginosa* 209 and *E. coli* carrying R135; the data of Le Goffic et al. (7) on *E. coli* R176/LA290 suggest that the enzyme in *P. aeruginosa* PST is different from Le Goffic's AAC(3)-II.

Characteristics of the acetylating enzyme in *P. aeruginosa* PST. To provide a more direct comparison of the acetylating enzymes from various resistant strains, we have compared the activity of cell-free extracts of *P. aeruginosa* PST with those of *P. aeruginosa* 209 and *E. coli* R135/C600. The results in Table 2 show that the acetylating enzyme in *P. aeruginosa* PST differs in substrate range compared with the other enzymes. As has been described previously (8), the 3-*N*-acetylating enzymes in *P. aeruginosa* 209 and *E. coli* R135/C600 are very similar. Although we have not assayed the 3-*N*-acetylating enzyme described by Le Goffic et al. (7), comparison with their data (shown in Table 2) indicates that *P. aeruginosa* PST does not contain their AAC(3)-II.

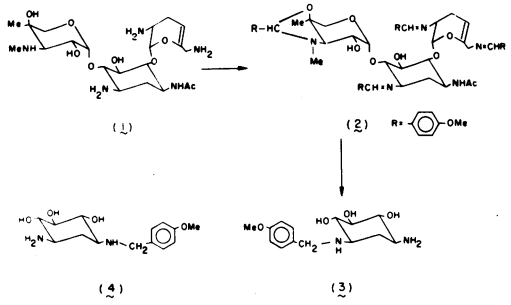

FIG. 1. *Degradation of N-acetylsisomicin.*

TABLE 1. *Resistance spectra of resistant strains expressed as minimal inhibitory concentrations (MIC) after 24 h of incubation*

Antibiotic	MIC (μg/ml)			
	P. aeruginosa PST	*P. aeruginosa* 209	*E. coli* R135/C600	*E. coli*[a] R176/LA290
Gentamicin C_1	>32	>32	>32	
Gentamicin C_{1a}	>32	>32	>32	
Sisomicin	>32	>32	>32	64
Tobramycin	>32	0.25	1	16
Amikacin (BB-K8)	1	2	4	0.25
Kanamycin A	>32	>32	4	8
Kanamycin B	>32	>32	2	8
Neomycin B	8	8	1	1
Paromomycin	>32	>32	4	4
Butirosin[b]	4	8	4	0.5

[a] Data taken from reference 7.

[b] The butirosin used in these determinations was a mixture of butirosin A and B in a ratio of 85:15.

TABLE 2. *Enzymatic activity (percentage relative to gentamicin C_1)*

Substrate	AAC (3)-I *P. aeruginosa*[a] 209	AC (3)-I *E. coli* R135/C600	*P. aeruginosa* PST	AAC (3)-II[b] *E. coli* R176/LA290
Gentamicin C_1	100	100	100	100
Sisomicin	96	95	185	80
Kanamycin A	0	0	64	15
Kanamycin B	26	18	84	30
Neomycin B	0	0	175	0
Paromomycin	0	0	100	0
Tobramycin	20	10	110	40
Amikacin (BB-K8)	0	0	0	0

[a] Data taken from reference 1.

[b] Data taken from reference 7.

Preparation and structure determination of sisomicin inactivated by the acetyltransferase of *P. aeruginosa* PST. The broad substrate activity of the acetyltransferase of *P. aeruginosa* PST did not allow us to make any conclusions concerning the site of modification by the enzyme. Accordingly, sisomicin was modified by incubation with an extract of *P. aeruginosa* PST and acetyl coenzyme A and purified by ion-exchange chromatography (Fig. 2). For comparison, *N*-acetylsisomicin produced by incubation of a cell-free extract from *E. coli* R135/C600 with sisomicin, in the presence of acetyl coenzyme A, was isolated following the same protocol.

Although the site of acetylation by the aminoglycoside-modifying enzyme of *E. coli* R135/C600 has been determined by other workers (8), we describe our structure determination since the method used to identify the site of modification, in addition to confirming the previous work, is novel and is, we believe, of more general applicability. The properties of this compound were compared directly with the modified sisomicin produced by incubation with a *P. aeruginosa* PST extract.

***N*-acetylsisomicin from *E. coli* R135/C600.** *N*-acetylsisomicin was a white amorphous solid, $[\alpha]_D^{25}$ + 138° (c 0.15, water); it was homogeneous by TLC using the lower phase of a chloroform-methanol-concentrated ammonium hydroxide (1:1:1) solvent system as developer (R_f = 0.32, R_f sisomicin = 0.25). The PMR spectrum showed absorptions at δ 1.19 (s, C-C$\underline{H}_3$), 1.92 (s, CO-C$\underline{H}_3$), 2.49 (s, N-C$\underline{H}_3$), 3.12 (s, N-C$\underline{H}_2$), 3.77 (q, J = 10.5, 4 Hz, H-2″), 4.04 (d, J = 12.5 Hz, H-5″ eq), 4.78 (broad multiplet, H-4′), 5.06 (d, J = 4 Hz, H-1″), 5.37 (d, J = 2.2 Hz, H-1′) ppm. The high-resolution mass spectrum

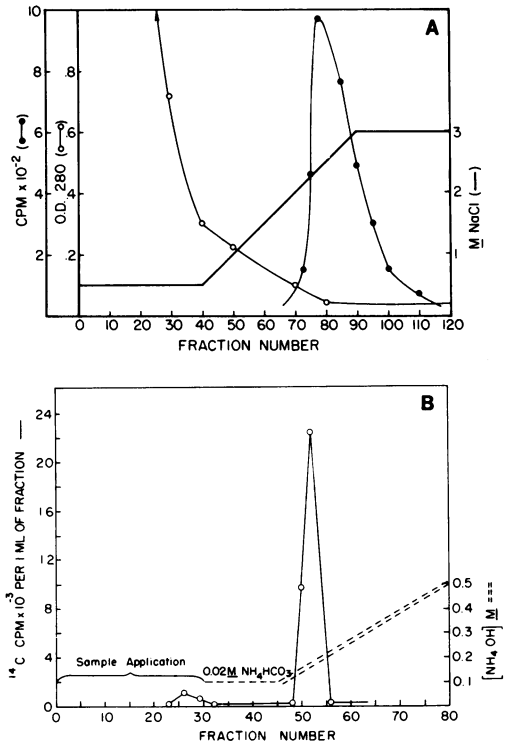

FIG 2. *Purification of sisomicin modified by an extract of P. aeruginosa PST (A). The modification of sisomicin by an extract of PST was conducted in a fashion analogous to the method described in the accompanying paper (4) for the modification of sisomicin by an extract of P. aeruginosa 3796. A total of 19.1 g of cells was washed and resuspended in 20 mM tris(hydroxymethyl)aminomethane-chloride-10 mM $MgCl_2$-25 mM NH_4Cl-10 mM KCl-2 mM dithiothreitol (pH 7.8) (buffer I), broken in a French pressure cell, and used to prepare an S-100 (40 ml) that was diluted to 60 ml with buffer I and employed in the modification of 50 mg of sisomicin. After 22 h of incubation at 30 C, a disk assay to determine the amount of remaining active sisomicin indicated that complete inactivation had occurred. The reaction mixture was centrifuged 20 min at 8,700 × g (4 C), and the supernatant was mixed with [^{14}C]acetylsisomicin tracer prepared by incubating 40 μl of PST S-100, 5 μl of 1 mg of sisomicin per ml, 5 μl of buffer 1, and 5 μl of 50 μCi of [^{14}C]S-acetyl coenzyme A per ml for 1 h at 30 C. The mixture was added to an Amberlite CG-50 column (100 to 200 mesh, sodium form, 2.5-cm diameter by 10-cm length), which was irrigated as described in the accompanying paper (4). Fractions ranging in volume from 5 to 12 ml were collected every 20 min. (B) Fractions 72 to 110 from (A) were combined, diluted to approximately 1 M NaCl with deionized water, and applied to and eluted from a second ion-exchange column, as described in the accompanying paper (4). Fractions 49 to 58 were pooled, concentrated, and submitted to structural analysis.*

showed ions at *m/e* 489·279 (21%, $C_{21}H_{39}N_5O_8$ M^+), 392·203 (5%, $C_{16}H_{30}N_3O_8$), 364·207 (19%, $C_{15}H_{30}N_3O_7$), 346·197 (66%, $C_{15}H_{28}N_3O_6$) (garosamine-*N*-acetyl-2-deoxystreptamine ions), 359·192 (28% $C_{15}H_{27}N_4O_6$), 341·183 (1.4%, $C_{15}H_{25}N_4O_5$), 331·198 (3.5%, $C_{14}H_{27}N_4O_5$), 313·187 (0.7%, $C_{14}H_{25}N_4O_4$) (unsaturated sugar-*N*-acetyl-2-deoxystreptamine ions), 233·113 (100%, $C_9H_{17}N_2O_5$), 215·103 (34%, $C_9H_{15}N_2O_4$), 205·119 (30%, $C_8H_{17}N_2O_4$), 187·108 (26%, $C_8H_{15}N_2O_3$) (*N*-acetyl-2-deoxystreptamine ions), 160·096 (63%, $C_7H_{14}NO_3$) (garosamine), 127·087 (21%, $C_6H_{11}N_2O$) (unsaturated sugar).

The *N*-acetylsisomicin (1) isolated from *E. coli* R135/C600 showed a single acetyl methyl resonance at δ 1.92 in its PMR spectrum. Its mass spectrum exhibited a molecular ion at *m/e* 489·279 corresponding to the expected molecular formula $C_{21}H_{39}N_5O_8$ for a mono-*N*-acetylsisomicin. Peaks in the mass spectrum at *m/e* 127 and *m/e* 160 were consistent with the presence of unsubstituted glycosyl units, whereas a series of intense ions at *m/e* 233, 215, 205, and 187 was consistent with a mono-*N*-acetyl-2-deoxystreptamine unit (3). The two series of pseudodisaccharide ions were also consistent with attachment of the acetyl residue to the 2-deoxystreptamine moiety. The acetyl residue was shown to be attached to *N*-3 by the degradation sequence outlined in Fig. 1. *N*-acetylsisomicin (1) was condensed with anisaldehyde to give the Schiffs base-oxazolidine derivative (2) (mass spectrum M^+ *m/e* 961) (2), which was reduced with sodium borohydride to the tetra-*N*-*p*-methoxybenzyl derivative, followed by hydrolysis with hydrochloric acid and chromatographic isolation of 1-*N*-*p*-methoxybenzyl-2-deoxystreptamine (3): melting point, 73 to 78 C, $[\alpha]_D^{26}$ − 37°, (c 0.1, water), CD: $[\theta]_{233}$ − 4,000, $[\theta]_{273}$ − 340; mass spectrum *m/e* 282 (M^+).

An authentic sample of 3-*N*-*p*-methoxybenzyl-2-deoxystreptamine (4) (5) was rechromatographed as described for (3) above and gave the following data: melting point, 80 to 85 C, $[\alpha]_D^{26}$ + 42° (c 0.15, water), CD:$[\theta]_{223}$ + 3,072, $[\theta]_{273}$ + 349. The two compounds (3) and (4) gave identical thin-layer chromatographs, low-resolution mass spectra, and identical PMR spectra of their hydrochloride salts. The position of the acetyl group was therefore at *N*-3 and the inactivation product is shown to be 3-*N*-acetylsisomicin (1).

3-*N*-acetylsisomicin from *P. aeruginosa* PST. 3-*N*-acetylsisomicin was a white amorphous solid, $[\alpha]_D^{26}$ + 132° (c 0.145, water). Its migration on TLC and its mass spectrum were identical to that of the sample prepared from *E. coli* R135/C600. The PMR spectra of the two

samples were very similar, the small differences observed being explicable by slight differences in carbonation of the sample. (On standing in the atmosphere, aminoglycosides absorb CO_2. Samples containing differing amounts of CO_2 show small differences in their nuclear magnetic resonance spectra.) Degradation of this sample was carried out as described for the *E. coli*-inactivated sample and also afforded 1-*N*-*p*-methoxybenzyl-2-deoxystreptamine (3) melting point, 68 to 75 C, $[\alpha]_D^{26}$ − 36.5° (c 0.1, water), CD: $[\theta]_{233}$ − 4,350, $[\theta]_{273}$ − 370; mass spectrum *m/e* 282 (M^+). The inactivation product is, therefore, 3-*N*-acetylsisomicin. TLC data for 3-*N*-acetylsisomicin are given in the accompanying paper (4).

It is noteworthy that the various *N*-acetylsisomicins differ very little from one another in their infrared and TLC properties. Although PMR data, with care, can be used to correctly identify some positions of mono-*N*-acetylation, this method fails to differentiate between 1- and 3-*N*-acetylsisomicin. Consequently, we feel that criteria used elsewhere (7) to establish the identity of 3-*N*acetylgentamicin C_{1a} as an inactivation product are inadequate, although we believe that this was indeed the product obtained.

DISCUSSION

It is now apparent that three of the known aminoglycoside-modifying activities, i.e., the 3′-*O*-phosphotransferases, the 6′-*N*-acetyltransferases, and the 3-*N*-acetyltransferases, exist in a variety of different forms with different substrate ranges. At the present time it is not possible to state whether these different forms are true isozymes, since detailed protein structure and sequence studies have not been carried out.

The new 3-*N*-acetylating activity described in this paper has the broadest substrate range of any aminoglycoside-modifying enzyme yet described, with the possible exception of the 6′-*N*-acetylating enzymes. The only agents not substrates for this enzyme are the 1-*N*-hydroxyaminobutyric acid derivatives butirosin and amikacin; the presence of the hydroxyaminobutyric acid substituent probably hinders the approach or attachment of the enzyme to the substrate.

Recently AAC(3)-I from *E. coli* R135/C600 has been purified to homogeneity, the first of the aminoglycoside-modifying enzymes to be obtained as a single component (9). For gentamicin C_{1a}, the enzyme has a K_m of 0.3 to 0.5 μm and a V_{max} of 3×10^8 international units/mol. The fact that strains carrying this enzyme are resistant to gentamicin and not tobramycin can be explained by the fact that the K_m for tobramycin is 10 times higher and the V_{max} is 6 times lower than those for gentamicin (D. B. Northrop, personal communication). The acetyltransferase that we describe in this article must possess different kinetic properties that are consistent with modification of gentamicin, tobramycin, and neomycin. However, it is surprising that this enzyme does not confer significant levels of neomycin resistance. This enzyme could have evolved from AAC(3)-I by mutational alteration, or the two enzymes may be derived from a common precursor.

Kojima et al. (6) have recently described the isolation of 3-*N*-acetylribostamycin during the fermentation of *Streptomyces ribosidificus*. We have detected *N*-acetylating activity in crude extracts of *S. ribosidificus*, but the nature of the modification has not yet been identified (J. Dowding, unpublished data). It thus seems likely that 3-*N*-acetylation is a bacterial resistance mechanism that, like 3′-*O*-phosphorylation, 6′-*N*-acetylation, and 2′-*N*-acetylation, is derived from and associated with the biosynthesis of the aminoglycoside antibiotics.

Attempts to transfer gentamicin resistance from *P. aeruginosa* PST to *P. aeruginosa* or *E. coli* recipients, using standard methods of gene transfer, have been unsuccessful. Treatment of *P. aeruginosa* PST with "curing" agents does not lead to loss of the acetyltransferase resistance determinant. Such data do not allow us to draw any conclusions as to the chromosomal or extrachromosomal nature of this determinant.

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