5-epi-Sisomicin and 5-epi-Gentamicin B: Substrates for Aminoglycoside-Modifying Enzymes That Retain Activity Against Aminoglycoside-Resistant Bacteria

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A number of bacterial strains, each possessing a different aminoglycoside-modifying enzyme, were examined for susceptibility to sisomicin and gentamicin B and the semisynthetic derivatives 5-epi-sisomicin and 5-epi-gentamicin B. Although strains possessing AAC(6') or APH(3') enzymes were equally resistant to the 5-epi compounds, those possessing AAC(3')I, ANT(2''), or AAC(2') enzymes were much more sensitive to the 5-epi derivatives. Analysis of partially purified aminoglycoside-modifying enzymes from the strains showed that the 5-epi compounds were substrates even for those enzymes found in susceptible strains [AAC(3')I, ANT(2''), and AAC(2')] However, a more detailed study of the enzymes showed that they had much increased K_m values for the 5-epi derivatives; the 5-epi compounds were much less effectively modified than the parent antibiotics. This confirms and extends the notion that enzymatic modification of aminoglycosides is not in itself sufficient to confer resistance to the drugs, but also that the modification must be efficient, as reflected in the K_m values.

Resistance to aminoglycoside antibiotics in clinical situations is determined predominantly by R-plasmid-encoded enzymatic modification of the antibiotics. These modifications involve O-phosphorylation, O-adenylylation, or N-acetylation of the aminoglycoside molecule (2). With the recognition of the biochemical basis of resistance, efforts have been directed towards the isolation and synthesis of compounds refractory to R-plasmid-mediated modification. This approach has been successful in providing butirosin (7), amikacin (9), and netilmicin (10)—aminoglycoside derivatives effective against certain classes of resistant bacteria since they are (apparently) not substrates for the enzymatic modifications involved.

Waitz et al. (12) have described a new semisynthetic derivative of sisomicin, 5-epi-sisomicin (Fig. 1), that has proved to be effective against a broad spectrum of aminoglycoside-resistant bacteria with known enzymatic mechanisms of resistance. Parallel studies by other workers have confirmed their conclusions (5, 8). It was assumed that the antibacterial activity of the 5-epi derivative was due to the failure of the enzymes present in the resistant strains to modify this compound.

However, it had not been demonstrated whether 5-epi-sisomicin was active against resistant bacteria due to the inability of the R-plasmid-mediated enzymes to modify this compound (as in the case of amikacin) or for some other reason (e.g., the modified product may still possess antibacterial activity).

Accordingly, we have compared gentamicin B with 5-epi-gentamicin B (T.L. Nagabhushan and A. B. Cooper, Program Abstr. 11th Int. Cong. Chemother., 19th Intersci. Conf. Antimicrob. Agents Chemother., Boston, Mass., abstr. no. 768, 1979) and sisomicin with 5-epi-sisomicin (Fig. 1) as substrates for several aminoglycoside-modifying enzymes and related these enzymatic data to the antimicrobial activity of the compounds.

MATERIALS AND METHODS

Bacterial strains used in these investigations are listed in Table 1.

Antibiotics were obtained from Peter Daniels of the Schering Corp., and radioactively labeled substrates were obtained from commercial suppliers.

Enzyme assays and preparations of crude extracts were performed largely as described previously (6). Routinely, 1-liter cultures were grown with shaking at 37°C in medium containing, per liter: 10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride, and 10 ml of glycerol. The cells were harvested in late logarithmic phase of growth, washed, and suspended in 10 ml of TMD buffer [50 mM Tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 7.8) containing 10 mM MgCl_2 and 1 mM dithiothreitol]. Cell disruption was achieved by passage through a French pressure cell in excess of 12,000 lb/in^2, and cell extracts
were obtained by centrifugation at 50,000 × g for 2 h.

 Partially purified preparations of each of the aminoglycoside-modifying enzymes were obtained by the following procedure (all steps at 4°C). Streptomycin sulfate was added to the cell extract to give a final concentration of 1.5%; this mixture was stirred for 15 min and then centrifuged at 15,000 × g for 15 min. The enzyme was precipitated from the supernatant by the addition of ammonium sulfate to 55% saturation; this mixture was stirred for 1 h and then centrifuged at 15,000 × g for 15 min. The precipitate was dissolved in 3.0 ml of TMD buffer. Sucrose was added to achieve a final concentration of 5% (wt/vol), and this solution was applied to a column of Sephadex G-100 (30.0 by 2.5 cm) equilibrated with TMD buffer. Elution with this same buffer was carried out at a flow rate of 10 to 20 ml per h and 60 2.0-ml fractions were collected and assayed for enzyme activity.

The aminoglycoside phosphotransferase (3') [APH(3')] and aminoglycoside adenylyltransferase (2') [ANT(2')] enzymes were assayed by the radiometric assay (6). The standard reaction mixture contained 1.0 mM adenosine triphosphate (either 32P or 35C labeled) and 0.12 mM aminoglycoside, all in TMD buffer. The reaction was started by addition of enzyme to give a total reaction volume of 50 μl. The mixture was incubated at 30°C for 15 min, and 20-μl samples were applied to 1-cm squares of Whatman P-81 phosphocellulose paper. These squares were washed three times in hot (70 to 80°C) distilled water and dried, and the radioactivity was determined with a scintillation counter. Kinetic determinations were performed in the same manner by varying the aminoglycoside concentration in the reaction mixture.

Rate measurements for the aminoglycoside acetyltransferase enzymes were determined by means of the spectrophotometric assay by using the DTNB reaction (1). The standard reaction mixture contained 0.15 mM acetyl-coenzyme A, 0.12 mM aminoglycoside, and 1.0 mM DTNB all in TMD buffer. The reaction was started by the addition of enzyme to give a total volume of 1.0 ml. The reaction was started at 25°C, and the enzyme rate was determined from the molar extinction coefficient of 1.36 × 104 for the thionitrobenzoate anion liberated in the reaction (4). Kinetic determinations on the acetyl-transferases were performed by using the above conditions by varying the concentration of the aminoglycoside substrates.

Minimal inhibitory concentrations (MICs) were determined by in vitro dilution tests by using 2 ml of nutrient broth (pH 7.2). An inoculum of 10⁴ to 10⁵ was used, obtained by suitable dilution of overnight cultures.

**RESULTS**

A comparison of the effectiveness of sisomicin, 5-epi-sisomicin, gentamicin B, and 5-epi-genta-
micin B against a number of bacterial strains is given in Table 1.

As described previously (5, 12) it can be seen that the 5-epi-compounds are highly potent antibiotics, even against many strains which are resistant to sisomicin or gentamicin B. It has been proposed that the 5-epi compounds may not be sensitive to attack by some of the aminoglycoside-modifying enzymes, thereby accounting for their antibiotic activity (8). However, when tested under the standard radioenzymatic assay conditions, the 5-epi compounds appeared just as prone to modification as gentamicin B or sisomicin (Table 2).

The APH(3') enzyme does not modify sisomicin or 5-epi-sisomicin, as these antibiotics do not have a 3' hydroxyl group. Similarly, the AAC(2') enzyme does not modify gentamicin B or its 5-epi derivative, as these compounds do not possess a 2' amino group. However, results with other enzyme-substrate combinations appear to be contrary to expectation since the modification enzymes listed above are able to modify the 5-epi compounds. Since this paradox might be due to differences in rate of modification that would not be detected by this simple assay, the kinetics of the modification of the four antibiotics using each enzyme, AAC(3)-I, ANT(2'), etc., were studied. The concentration of the aminoglycoside in the reaction mixture was varied while the second substrate was maintained at a fixed concentration. Figure 2 shows a typical double-reciprocal plot for the AAC(3)-I enzyme comparing gentamicin B and 5-epi-gentamicin B as substrates. It can be seen that with the use of high concentrations of aminoglycoside antibiotics (as in the standard assay) both gentamicin B and its 5-epi derivative are modified equally. However, at low substrate concentrations the 5-epi compound is very poorly modified compared with gentamicin B. This finding is reflected in the much higher $K_m$ value for 5-epi gentamicin B than for gentamicin B.

Similar studies were conducted for the four aminoglycosides with several modification enzymes, and the $K_m$ values are given in Table 3.

In an attempt to relate these values to physiological effectiveness of the drugs we have compared these $K_m$ determinations with the MICs. Tables 1 and 3 show a striking correlation between the effectiveness of the modification enzyme (as reflected in the $K_m$ value) and the degree of protection that it affords to the cell (as measured by the MIC).

**DISCUSSION**

The antibiotic resistance patterns obtained for each strain are very similar to those previously reported by other workers (5, 8, 12). We have found that the presence of an AAC(6') enzyme resulted in bacterial resistance to sisomicin, gentamicin B, and both 5-epi compounds. The presence of an APH(3') enzyme gave resistance to gentamicin B and its 5-epi derivative. Surprisingly, however, we found that some enzymes, although able to modify the 5-epi compounds, did not confer resistance to these compounds. This apparent discrepancy was resolved by a more detailed study of the enzymes which showed significant differences in the efficacy with which the antibiotics are modified.

![Fig. 2. Determination of the $K_m$ of gentamicin B (•) and $K_m$ of 5-epi-gentamicin B (○) for the AAC(3)-I enzyme by double-reciprocal plot analysis.](http://aac.asm.org/)

**TABLE 2. Comparison of the specific activity of each aminoglycoside-modifying enzyme for aminoglycoside substrates**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sisomicin</th>
<th>5-epi-sisomicin</th>
<th>Gentamicin B</th>
<th>5-epi-gentamicin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANT(2')</td>
<td>124,400</td>
<td>135,300</td>
<td>164,000</td>
<td>149,900</td>
</tr>
<tr>
<td>APH(3')</td>
<td>300</td>
<td>400</td>
<td>236,000</td>
<td>270,800</td>
</tr>
<tr>
<td>AAC(3)-I</td>
<td>0.16</td>
<td>0.24</td>
<td>0.19</td>
<td>0.26</td>
</tr>
<tr>
<td>AAC(6')</td>
<td>0.07</td>
<td>0.07</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>AAC(2')</td>
<td>0.09</td>
<td>0.10</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Standard assay conditions were used; specific activity is expressed as counts per minute per milligram of protein for the ANT(2') and APH(3') enzymes and as micromoles of compound acetylated minute per milligram of protein for the acetylating enzymes.
The ANT(2\"), AAC(3)-I, and AAC(2\') enzymes have very much higher \( K_a \) values for the modification of the 5-epi derivatives than they do for the parent compounds. This shows that that epimerization of the hydroxyl group at the 5-position of the 2-deoxystreptamine ring results in a substantially reduced rate of enzymatic modification; this reduction in modification rate must be responsible for sensitivity in strains possessing these enzymes. Thus, the ability of an R-plasmid-encoded resistance enzyme to catalyze antibiotic modification is not necessarily sufficient to determine resistance to aminoglycosides, but the modification must proceed at a certain minimum rate. This minimum rate cannot be ascertained with the standard enzymatic assay, with the result that the striking correlation between effective antibacterial concentration (MIC) and enzymatic activity (\( K_a \)) was not obvious from previous experiments.

In contrast to the differences noted above, the AAC(6\') enzyme had similar \( K_a \) values for all four compounds tested, and APH(3') also had a low \( K_a \) value for both gentamicin B and 5-epi-gentamicin B. Thus, epimerization at the 5-position of 2-deoxystreptamine does not significantly affect the activity of all enzymes against their antibiotic substrates. However, different changes in aminoglycoside structure may well be effective in causing the appropriate reduction in enzymatic activity in other cases.

Since the establishment of aminoglycoside resistance by plasmid determined modifying enzymes is so dependent on the \( K_a \) value for the antibiotic, it seems reasonable to propose that the development of competitive inhibitors for the aminoglycoside-modifying enzymes could have important chemotherapeutic application. Such inhibitors need not in themselves have antibacterial activity, but used in combination with other aminoglycosides they could sufficiently reduce the \( K_a \) value for the drug to render resistant bacteria susceptible to antibiotic treatment.

Considerable success has been reported in the case of inhibitors of \( \beta \)-lactamase enzymes that can be used to potentiate the activity of \( \beta \)-lactam antibiotics against resistant organisms. Clavulanic acid is a typical example; this \( \beta \)-lactam interacts with \( \beta \)-lactamases by binding to the active site, thereby impeding further activity (11). In the light of our findings it should be profitable to examine additional aminoglycosides to find a compound which will act as a powerful inhibitor of the aminoglycoside-modifying enzymes. It is significant that the single change in the 5-epi compounds can affect at least three different forms of aminoglycoside modification—in fact, three of the most widely distributed and important in terms of resistance to this class of drugs. Thus, although there are several known types of aminoglycoside modification, these results lend hope to the thesis that other changes may also prove to be effective. In addition to synthetic derivatives, a search for naturally occurring inhibitors might be profitable, especially since the spectrophotometric assay for the aminoglycoside acetyltransferases can provide such a facile assay system.

Our results confirm and extend earlier proposals with respect to the mechanism of resistance to aminoglycoside antibiotics determined by R-plasmid encoded modifying enzymes. It is obvious that the rate and extent of aminoglycoside modification are of paramount importance in the establishment of resistance. If the \( K_a \) for an antibiotic substrate is increased by as little as 10-fold the organism becomes susceptible (note that the 5-epi compounds have \( K_a \) values 100 times higher in some cases). Whether an effective rate of modification of an aminoglycoside antibiotic is required to set up some kind of permeability block to stop the uptake of antibiotic into the cell, or if the rate of modification simply has to be sufficient to counteract the inefficient entry of the antibiotic into the cell, remains unclear (3).

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LITERATURE CITED