# BIOTRANSFORMATION, A NEW APPROACH TO AMINOGLYCOSIDE BIOSYNTHESIS. I. SISOMICIN

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Utilizing a mutant of *Micromonospora inyoensis* which requires the addition of 2-deoxystreptamine for sisomicin production, the bioconversion of 2-deoxystreptamine containing pseudodisaccharides and pseudotrisaccharides into sisomicin was demonstrated. The trisaccharides tested were structurally related minor components found in the sisomicin or gentamicin fermentations. Based upon the specificity of the structural configuration of those compounds which were converted to sisomicin versus those which were not, a pathway for the biosynthesis of sisomicin is proposed.

Deoxystreptamine-negative (DOS$^-$) mutants from a number of aminoglycoside-antibiotic-producing microorganisms have been used to produce novel antibiotics by the addition of 2-deoxystreptamine analogues to the fermentation broth.[1~7] Testing of 2-deoxystreptamine containing pseudodisaccharides for conversion into antibiotics has been reported in three cases with such mutants[3,4,7], and in only one of these studies was a conversion noted[3]. Moreover, the modification of complete aminoglycoside antibiotics by the producing organisms themselves, such as the N-methylation and phosphorylation of streptomycin[8,9], the N-carboxymethylation of ribostamycin[10], and the acetylation of kanamycin, neomycin and gentamicin[11] has also been reported.

This communication is an expansion of the studies with the DOS$^-$ mutant of *Micromonospora inyoensis*, as described by TESTA *et al*,[6] to include the testing of aminocyclitol pseudodisaccharides and pseudotrisaccharides for conversion into sisomicin. From the results of those compounds converted, a biosynthetic pathway for sisomicin is proposed.

## Materials and Methods

### Organism and Culture Conditions

*Micromonospora inyoensis* (1550F), a 2-deoxystreptamine-negative (DOS$^-$) mutant, was used in this study. The mutant does not produce any antibiotic except with the addition of 2-deoxystreptamine or certain 2-deoxystreptamine analogues. The mutant was grown in a medium consisting of (g/liter); yeast extract, 5 g; beef extract, 3 g; tryptose, 5 g; starch, 24 g; dextrose, 5 g; and calcium carbonate, 4 g; for 3 days at 35°C on a rotary shaker. Inocula prepared in this fashion were used at 5 % (v/v) for fermentations in the following medium (g/liter); soybean meal, 35 g; dextrin, 50 g; dextrose, 5 g: calcium carbonate, 7 g; and cobalt chloride ($7H_2O$) 0.24 mg. Fermentations were carried out on a rotary shaker at 300 rpm for 6~7 days at 28°C.

Compounds tested for bioconversion were supplied by the Antiinfective and Antibiotic Chemistry Department of Schering Corporation. All were added at 0 hour or after 24 hours of fermentation depending upon their toxicity to the mutant. Initial studies were performed

with compounds added at 100 $\mu$g/ml. The fermentations were allowed to proceed normally after addition of the material.

Detection of Transformation Products

Oxalic acid was added to the whole broth to precipitate calcium ions, and the pH of the fermentation was further adjusted to 2 with sulfuric acid to release the antibiotic from the mycelium. After filtration, the clarified broth was neutralized with ammonium hydroxide, the antibiotic was adsorbed on Amberlite IRC-50 (20~50 mesh) ion-exchange resin in the $NH_4^+$ cycle, and the spent broth was discarded.

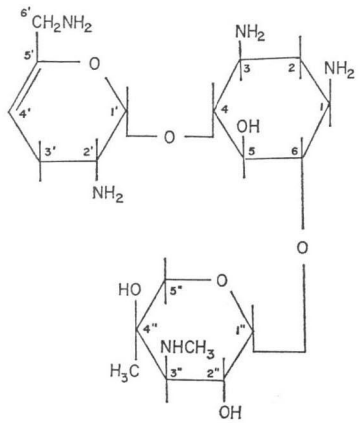
The antibiotic was eluted from the resin with 2 N ammonium hydroxide, and the eluate was evaporated to dryness. The dried material was then dissolved in distilled water to the desired concentration.

Transformation products were detected by paper and thin-layer chromatography of acidified broth extracts and concentrated resin eluates in solvent systems consisting of the lower phase of chloroform - methanol - 17 % ammonium hydroxide at a ratio of 2:1:1 (v/v) for the former method and 1:1:1 (v/v) (concentrated ammonium hydroxide) for the latter. The paper chromatograms were developed further by both spraying with ninhydrin reagent with subsequent determination of Rf's and the characteristic color development as described for sisomicin[12], and also by bioautography against *Staphylococcus aureus* FDA 209 P. Thin-layer chromatograms were visualized with iodine treatment. All products were checked against reference and control samples. Purified sisomicin for mass spectral analysis was prepared using a procedure described previously[12].

## Results and Discussion

Screening 2-deoxystreptamine (2-DOS) analogues for utilization by a DOS$^-$ mutant of *M. inyoensis* was expanded into testing 2-deoxystreptamine containing pseudodisaccharides for conversion into sisomicin (Fig. 1). The pseudodisaccharides tested are given in Fig. 2. Paromamine (**1**), and to a lesser extent neamine (**2**), were transformed by the mutant into sisomicin (Fig. 3). Gentamine JI-20 B (**3**) (a 6′C-methyl neamine), gentamine $C_1$a (**4**) (a 3′, 4′-dideoxyneamine), and gentamine $C_1$ (**5**) (a 6′C-methyl, 6′N-methyl gentamine $C_1$ a) were not transformed into sisomicin or any other detectable

Fig. 1. Structure of sisomicin.



Sisomicin

Fig. 2. Pseudodisaccharides tested for biotransformation.

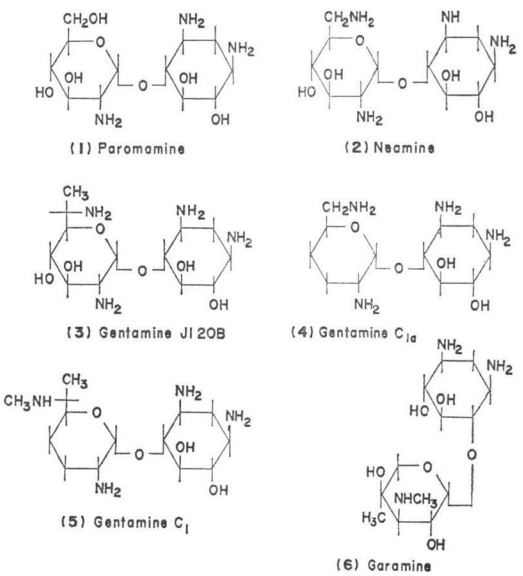
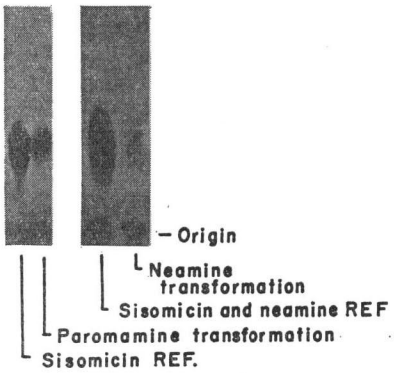


Fig. 3. Bioautogram of paromamine and neamine biotransformation products.
Solvent system: chloroform - methanol - 17% ammonium hydroxide (2:1:1). Whatman #1 paper,

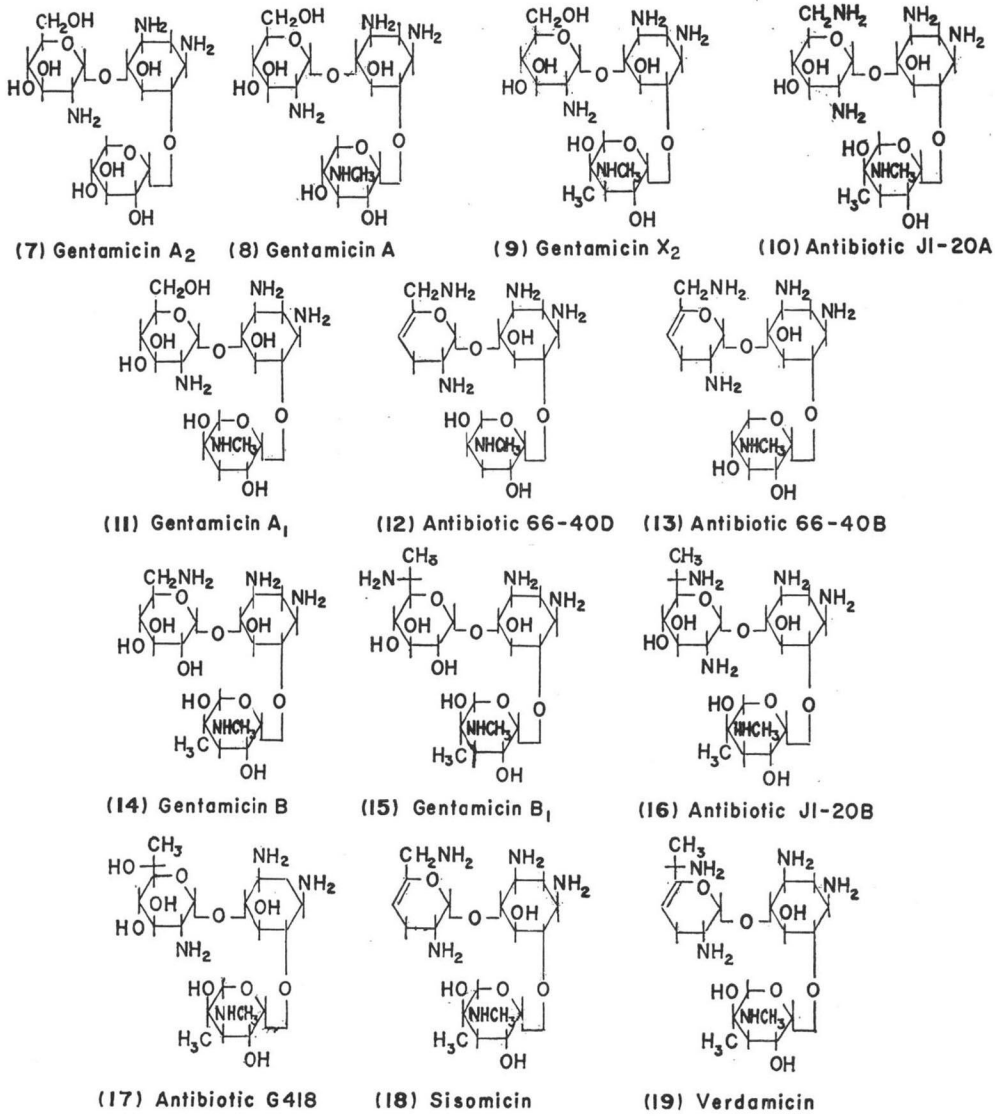



antibiotic. Garamine (**6**), a disaccharide portion of the sisomicin molecule, also was not transformed. The specificity demonstrated by the mutant as to the compounds utilized indicates that the organism most likely is not able to hydrolyze random 2-DOS containing disaccharides and then utilize the free 2-DOS for synthesis into sisomicin. Also, since paromamine and neamine are transformed into sisomicin, it seems probable that they, rather than garamine, are precursors to sisomicin.

The conversion noted with certain pseudodisaccharides prompted the screening of pseudotrisaccharides for transformation by the mutant (Fig. 4). The compounds tested are all coproduced in the gentamicin or the sisomicin fermentations[13~18]. Gentamicins $A_2$[16] (**7**), A (**8**) and $X_2$ (**9**), paromamine containing pseudotrisaccharides, and JI-20A[17] (**10**), a neamine containing pseudotrisaccharide, were all transformed into sisomicin (Fig. 5). Gentamicin A was available in sufficient quantity to transform into the required amount of antibiotic for isolation and chemical characterization. The antibiotic was shown to be sisomicin, based on mass spectral analysis[15], the characteristic ninhydrin color, and chromatographic identity with sisomicin. Gentamicin $A_1$[18] (**11**), a 4″ epimer of gentamicin A, was transformed into 66-40D (**12**)[14], a 4″ demethyl sisomicin. The gentamicin $A_1$ used in these studies was found to be contaminated with a small amount of gentamicin A, as determined by TLC, and this most likely is the reason for the presence of the sisomicin (Fig. 6) and not the conversion of 66-40D to sisomicin since no conversion is noted when 66-40D is tested. This observation indicates that there is a marked preference for sisomicin biosynthesis. Antibiotic 66-40B (**13**)[14], a 4″ demethyl xylo form of sisomicin, was transformed into sisomicin. Pseudotrisaccharides tested but not transformed into any other detectable antibiotic were gentamicin B (**14**), $B_1$ (**15**), JI-20B (**16**), antibiotic G-418 (**17**), 66-40D (**12**), and sisomicin (**18**) itself.

From the results obtained it is possible to draw several conclusions: (1), The mutant is able to convert either an aminocyclitol (2-deoxystreptamine), pseudodisaccharides (paromamine and neamine), and pseudotrisaccharides (gentamicin A, *etc*), into sisomicin. (2) The compounds tested were most likely not hydrolyzed to yield free 2-DOS which would then be used by the organism. This is demonstrated by the specificity of the mutant both as to the type of compounds that were converted and the products formed. For example, if hydrolysis were the case, gentamicin $A_1$ should yield a product identical to those of gentamicin A, *i.e.* mostly sisomicin, rather than the large quantity of 66-40D as noted in Fig. 6. (3) The organism appears to be incapable of altering compounds through demethylation at the 6′ position or amination at the 2′ position. This was demonstrated for the former by the inability of the organism to convert the gentamine JI-20B (**3**), antibiotic JI-20B (**16**), or antibiotic G-418 (**17**); and for the latter by the inability to convert either gentamicins B (**14**) or $B_1$ (**15**).

Fig. 4. Pseudotrisaccharides tested for biotransformation.




Based upon the results of these transformations, as evidenced by chromatographic comparisons, the branched pathway for sisomicin biosynthesis as shown in Fig. 7 is proposed.

It is realized that there are limitations in the use of chromatography for identification and that several other intermediates may be involved. However, this is presented as a working hypothesis to expand upon with future experimentation and characterization of the compounds produced.

Additional suggestive evidence for the involvement of gentamicin A in the pathway to sisomicin is that in the production of mutamicin 2, *i.e.*, by the addition of 2, 5-dideoxystreptamine to the DOS$^-$ mutant, mutamicin 2A, the dideoxystreptamine containing analogue of gentamicin A, has been isolated and identified[6]. Also several mutants have been isolated from the gentamicin and sisomicin producing organisms that yield increased quantities of the more

Fig. 5. Bioautogram of pseudotrisaccharides and 2-deoxystreptamine biotransformation products.
Solvent system: chloroform - methanol - 17 % ammonium hydroxide (2:1:1)

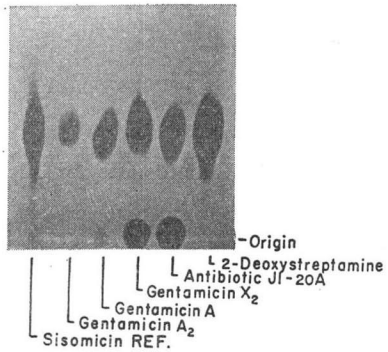



Fig. 6. Bioautographic comparison of gentamicins A and $A_1$ biotransformation products.
Solvent system: chloroform - methanol - 17% ammonium hydroxide (2:1:1).

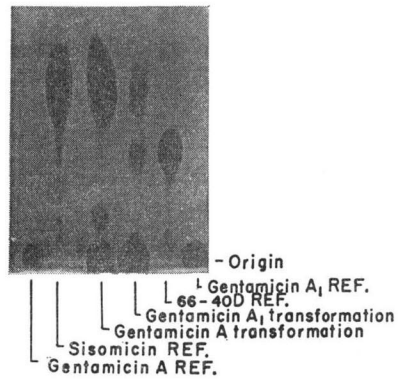
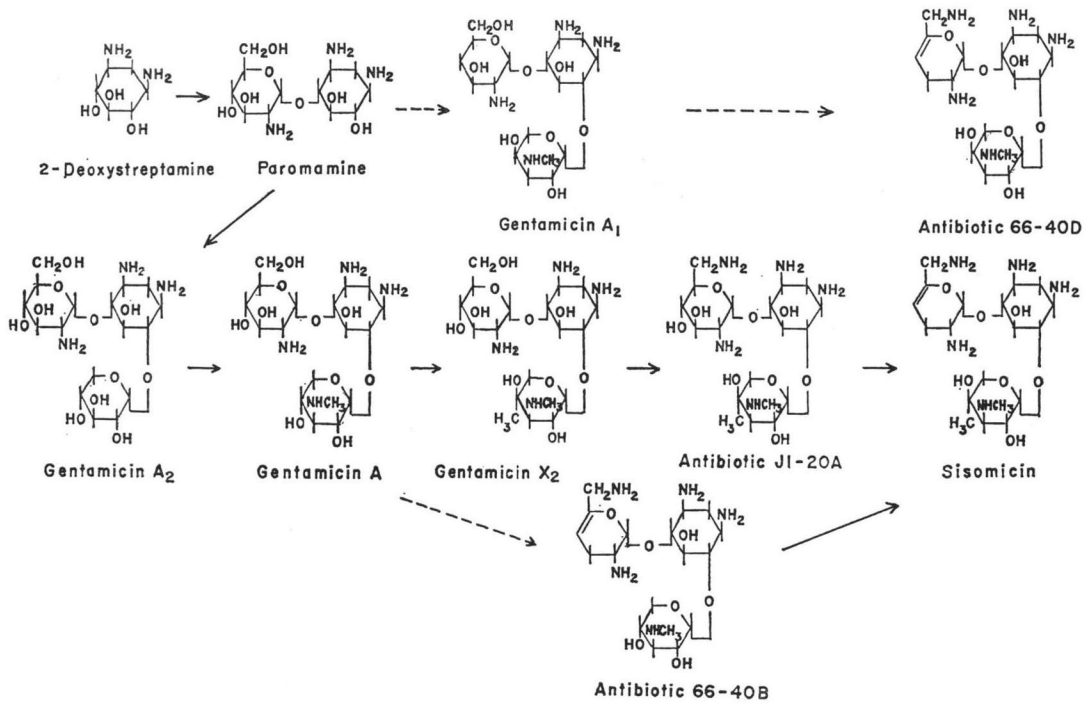



Fig. 7. Proposed pathway for sisomicin biosynthesis.
Broken lines represent steps via unidentified intermediates.




polar minor components which were used in the studies.

Based upon these results, the potential exists for similar studies to be carried out with other aminoglycoside antibiotic producing organisms for which DOS$^-$ mutants have been described such as neomycin, kanamycin, ribostamycin and butirosin. No bioconversion of several disaccharides was noted in recent studies with DOS$^-$ mutants from *Bacillus circulans*,[4]

*Streptomyces fradiae*, *S. kanamyceticus*, and *S. rimosus* forma *paromomycinus*,[7] but bioconversion of disaccharides was noted with the DOS⁻ mutant from *S. ribosidificus*[3]. The formation of structurally related co-produced components has been described with several other aminoglycoside antibiotic producing cultures[19~22], and the technique used to provide the proposed pathway for sisomicin biosynthesis may be applicable in these cases.

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