BIOTRANSFORMATION, A NEW APPROACH TO AMINOGLYCOSIDE BIOSYNTHESIS: II GENTAMICIN

R.T. TESTA and B.C. TILLEY

Schering Corporation, Bloomfield, New Jersey 07003, U.S.A.

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Utilizing a paromamine-producing mutant of *Micromonospora purpurea* blocked in the production of gentamicin, bioconversion of various minor gentamicin components into the gentamicin C complex was demonstrated. The compounds tested were structurally related to the gentamicin C's and are found as minor components in the gentamicin fermentation. Based upon the bioconversions detected, a branched pathway for the biosynthesis of the gentamicin C components is proposed.

Gentamicin is a broad-spectrum, aminoglycoside antibiotic complex, produced by *Micro monospora purpurea* which consists of 3 major components and numerous minor ones[1,2,3]. The major components are gentamicin C_1, C_2 and C_{1a} which differ from each other by the degree of methylation at the 6′ position[4]. A minor component closely related to the gentamicin C's is gentamicin C_{2b}, a 6′-N-methyl C_{1a}[5]. Several other minor components such as gentamicins A, A_1, A_2, A_3, B, X_2[2,3,6] and antibiotics JI-20A and JI-20B[7] have been described which are structurally related to the gentamicin C's.

Several factors, such as the isolation of various mutants which produce large quantities of the minor components[5,7], the results of the incorporation of methyl-^{14}C-L-methionine[8], and the build-up of gentamicin A concomitant with poorer gentamicin C production when cobalt is omitted from the fermentation medium[9] suggest that these minor components may be precursors to the gentamicin C's. The bioconversion of many of these components to the gentamicin C components by a mutant of *M. purpurea* blocked in gentamicin formation was used to test this hypothesis. Based upon the bioconversions detected, a branched pathway for the gentamicin C's is proposed.

Meterials and Methods

Organism and Culture Conditions

Micromonospora purpurea Paro 346, a mutant which produces paromamine and is blocked in gentamicin formation was used in this study. The mutant was isolated in our laboratories by Dr. JAN ILVASKY. The organism was grown in a medium consisting of (g/liter); yeast extract, 5; beef extract, 3; tryptose, 5; starch, 24; dextrose, 5; and calcium carbonate, 4; 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; dextrin, 50; dextrose, 5; calcium carbonate, 7; and cobalt chloride·7 H_2O, 0.00024. 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 after 24 hours of fermentation at 500 mcg/ml base equivalents. 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 ion-exchange resin (20~50 mesh) in the NH_4^+ cycle, and the spent broth was discarded.

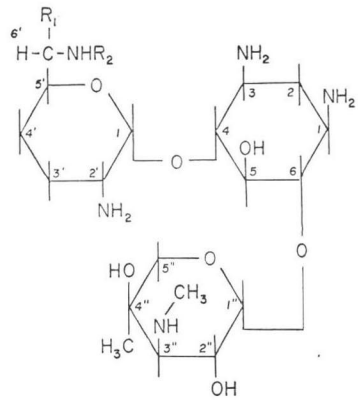
The antibiotic was eluted from the resin with 2 N ammonium hydroxide, and the eluate was evaporated to dryness. The dried material was then dissolved in distilled water to the desired concentration.

Transformation products were detected by paper and thin-layer chromatography of acidified broth extracts and concentrated resin eluates using 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 chromatographic medium and 1 : 1 : 1(v/v) (substituting concentrated ammonium hydroxide) for the latter. Detection of antibiotic zones on the paper chromatograms was done by bioautography against *Staphylococcus aureus* ATCC 6538P. All products were checked against reference and control samples.

Results and Discussion

The biotransformation of several antibiotic molecules, including gentamicins A and X_2, into sisomicin by a deoxystreptamine-negative mutant of *M. inyoensis* was used by TESTA and TILLEY[10] to propose a pathway for sisomicin biosynthesis. In a similar fashion, the transformation of minor components of the gentamicin fermentation by the Paro 346 mutant was used to determine a possible biosynthetic pathway for the gentamicin C's (Fig. 1). This mutant of *M. purpurea* is blocked in gentamicin formation and produces the disaccharide paromamine (Fig. 2).

Fig. 1. Structure of the gentamicin C's

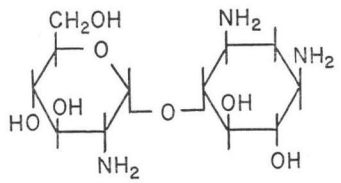


Gentamicin C_1 R_1= CH_3 ; R_2= CH_3

Gentamicin C_2 R_1= CH_3 ; R_2= H

Gentamicin C_{1a} R_1= H ; R_2 = H

Fig. 2. Structure of paromamine



Gentamicin C_1 was not transformed by the mutant into any other detectable active component, while gentamicin C_2 was transformed into gentamicin C_1. Gentamicin C_{1a}, however, was transformed into gentamicin C_{2b} (Fig. 3A, 3B and 3C). Both of these steps require an N-methylation step at the 6′ position. This suggests that there may be 2 different pathways to the gentamicin C's.

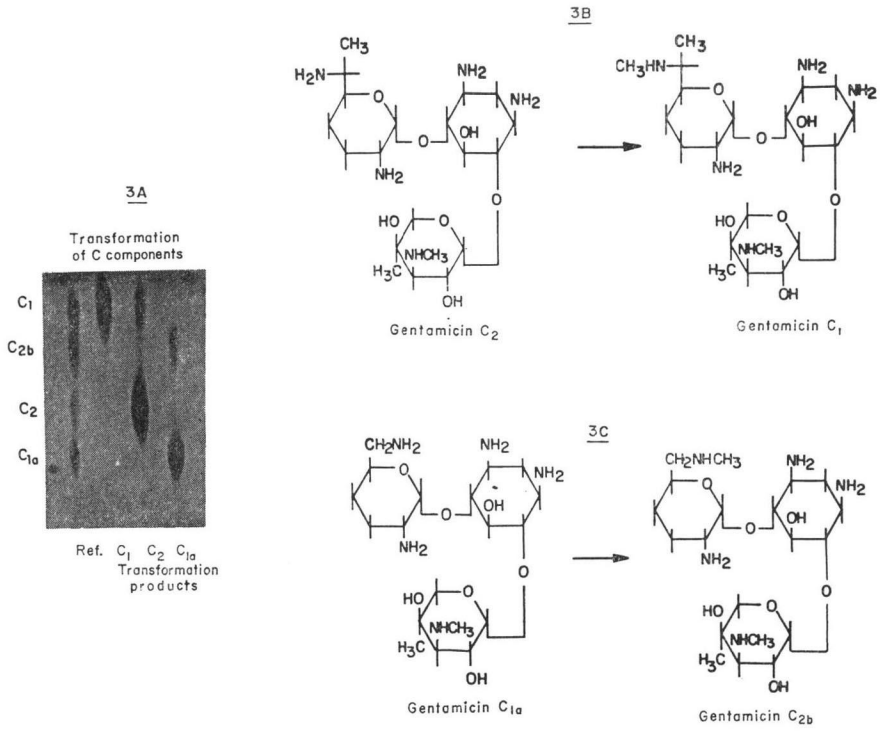
Other gentamicins and related compounds isolated from the fermentation of *M. purpurea* were tested to determine which, if any, of these may be precursors to the gentamicin C's. Antibiotic JI-20A, a 6′-amino gentamicin X_2, and JI-20B, a 6′-C-methyl JI-20A, both produced by a mutant of *M. purpurea*, were transformed but not to the same products (Fig. 4A, 4B and 4C): Antibiotic JI-20A was transformed to gentamicins C_{1a} and C_{2b} (Fig. 4A and 4B), while

Fig. 3A. Biotransformation products of the gentamicin C components. Bioautographic comparison.

Solvent system: chloroform - methanol - 17 % ammonium hydroxide (2 : 1 : 1), lower phase

Fig. 3B. Structural changes occurring in the biotransformation of gentamicin C_2 to C_1

Fig. 3C. Structural changes occurring in the biotransformation of gentamicin C_{1a} to C_{2b}



antibiotic JI-20B was transformed to gentamicins C_2 and C_1 (Fig. 4A and 4C). Antibiotic G-418[11], structurally related to antibiotic JI-20B, was also transformed into gentamicin C_2 and C_1 (Fig. 5A and 5B). These results are also suggestive of the involvement of a branched pathway for the formation of the gentamicin C's.

Gentamicins A and X_2 are paromamine-containing antibiotics produced in the gentamicin fermentation. Both of these antibiotics were transformed by the mutant into gentamicins C_{1a}, C_2 and C_1 (Fig. 6A, 6B). Therefore, it appears that these antibiotics are precursors to the C's before the branch point. Gentamicins B and B_1[2,3] (Fig. 7), also produced in the gentamicin fermentation, were not transformed by the mutant into any other detectable antibiotics. Since the gentamicin B's lack the 2′-NH_2 group that is common to paromamine and all of the compounds tested, this suggests that they are not precursors to the gentamicin C's by this route and perhaps another analogous but independent pathway is involved in their synthesis or conversion.

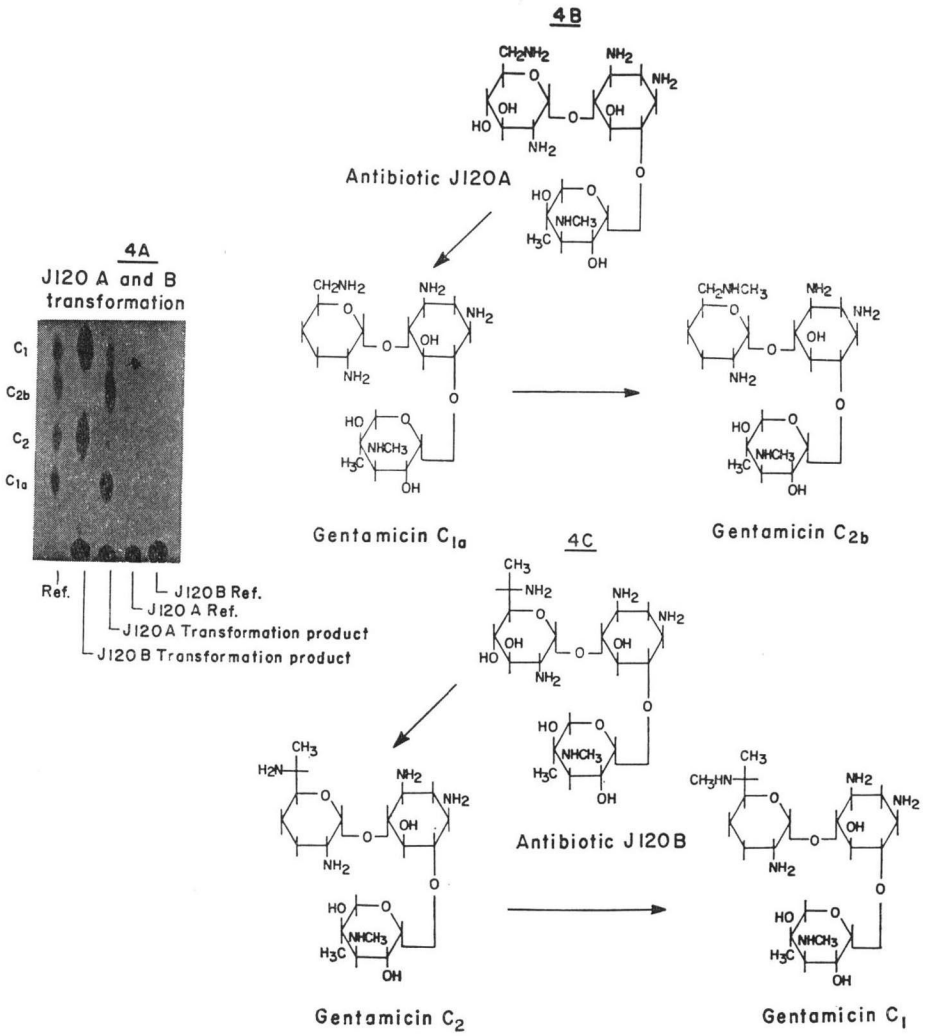
Based upon the transformation results, as evidenced by chromatographic comparisons, and the structures of the compounds tested, a branched pathway for the formation of the gentamicin C components is proposed (Fig. 8). Gentamicin X_2 appears to be the site of the branch point, for compounds added after that point lead either to the formation of gentamicins

Fig. 4A. Biotransformation products of antibiotics JI-20A and JI-20B. Bioautographic comparison.

Solvent system: chloroform - methanol - 17 % ammonium hydroxide (2 : 1 : 1), lower phase

Fig. 4B. Structural changes occurring in the biotransformation of antibiotic JI-20A to gentamicin C_{1a} and C_{2b}

Fig. 4C. Structural changes occurring in the biotransformation of antibiotic JI-20B to gentamicin C_2 and C_1



C_{1a} and C_{2b} or to gentamicins C_2 and C_1. It is realized that there may be other intermediates involved we have not tested or detected. Further characterization and experimentation are continuing in this area to confirm the results obtained using the chromatographic procedures.

The products formed by these transformations demonstrate that the mutant is able to carry out the enzymatic steps leading to the gentamicin C components (Fig. 8). These include C-methylations, epimerizations, dehydroxylations and N-methylations. Using this bioconversion system several factors affecting specific steps in gentamicin biosynthesis can be studied.

Fig. 5A. Bioautogram of antibiotic G-418 biotransformation products
Solvent system: chloroform - methanol - 17 % ammonium hydroxide (2 : 1 : 1), lower phase

Fig. 5B. Structural changes occurring in the biotransformation of antibiotic G-418 to gentamicin C_2 and C_1

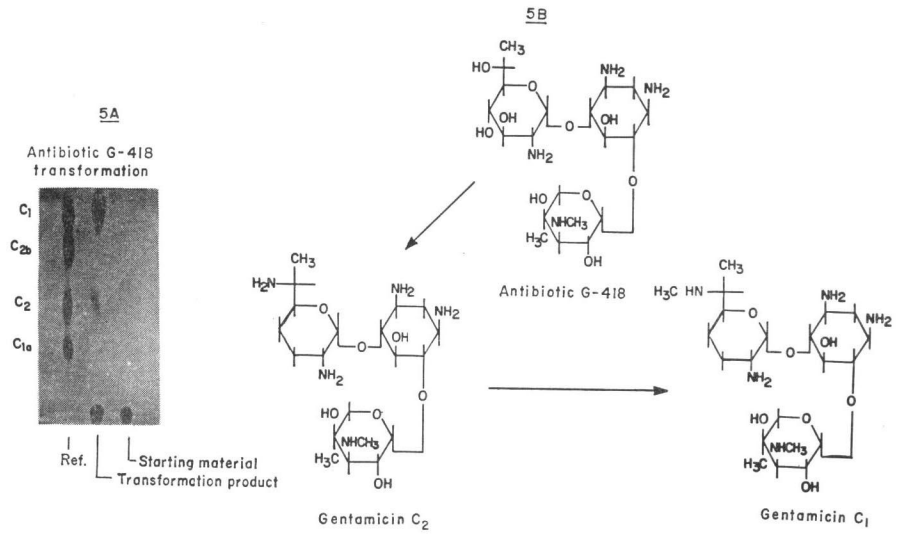


Fig. 6A. Biotransformation products of gentamicins A and X_2. Bioautographic comparison. Solvent system: chloroform - methanol - 17 % ammonium hydroxide (2 : 1 : 1), lower phase

Fig. 6B. Structural changes occurring in the biotransformation of gentamicins A and X_2 to the gentamicin C's

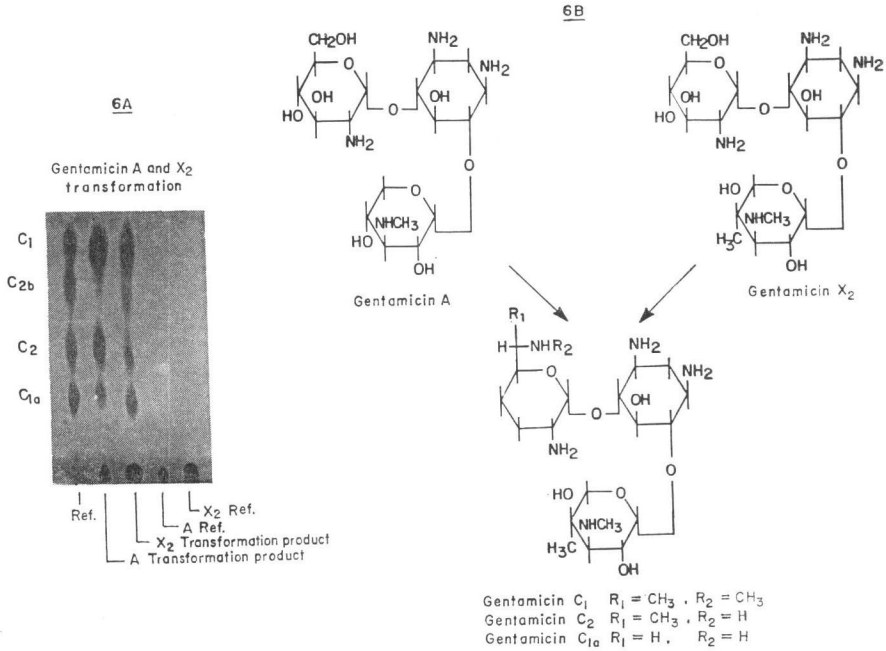
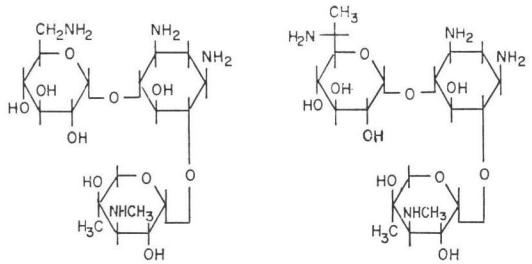


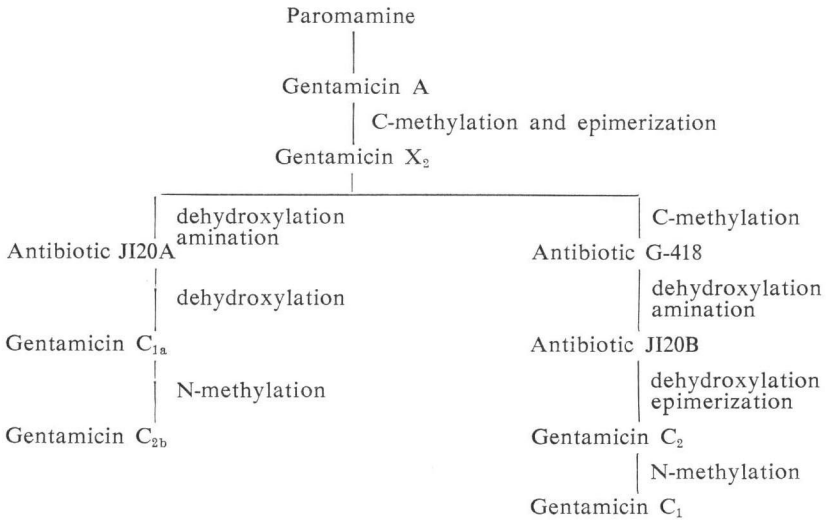
Fig. 7. Structure of gentamicins B and B_1.



TILLEY *et al.*[9], have been able to demonstrate which of the transformation steps require cobalt by this technique.

From these results, as well as those in a previously reported study utilizing a deoxystreptamine-negative mutant of the sisomicin producer, similar studies utilizing blocked mutants of other aminoglycoside antibiotics may be useful in learning more about the biosynthesis of these important antibiotics.

Fig. 8. Proposed biosynthetic pathway



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