Biotransformation of Sisomicin to Gentamicin C$_{2b}$

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Sisomicin was transformed to gentamicin C$_{2b}$ by *Micromonospora rhodorangea* NRRL 5326. The mechanisms involved in the biotransformation are the 6'-*N*-methylation and the (4'-5')-reduction. The progression of the methylation was followed by the isotope technique, but the reduction reaction was not monitored.

Gentamicin C$_{2b}$ is 6'-*N*-methylgentamicin C$_{1a}$. This antibiotic is coproduced, as one of minor components, in the standard gentamicin fermentation. Kershner (Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1971) first described its presence in trace amounts in gentamicin preparations. Daniels and co-workers (2) reported its isolation, purification, and characterization from fermentation both of a *Micromonospora purpurea* mutant, which produced gentamicin C$_{1a}$ as a major product and gentamicin C$_{2b}$ as a minor product. Unlike the parental gentamicin-producing strain, however, the mutant strain did not produce any detectable amounts of gentamicins C$_1$ and C$_2$. Okachi and co-workers (6) reported on the isolation of sagamicin from *Micromonospora sagamiensis*, which was found to be identical to gentamicin C$_{2b}$ (2).

Transformation of gentamicin C$_{1a}$ or sisomicin to gentamicin C$_{2b}$ has been achieved by an *M. purpurea*-blocked mutant, SC 1124 (B. K. Lee and R. G. Condon, unpublished data). Whereas the transformation of gentamicin C$_{1a}$ to C$_{2b}$ would require a single enzymatic reaction, 6'-*N*-methylation, that of sisomicin to C$_{2b}$ would require (4'-5')-reduction as an additional enzymatic reaction. *Micromonospora rhodorangea* NRRL 5326 has been shown to produce antibiotic G-418 (6'-*C*-methylgentamicin X$_2$). Since it was thought this strain might lack the (4'-5')-reduction activity, we chose this strain rather than *M. purpurea* SC 1124 to transform sisomicin to 6'-*N*-methylsisomicin (antibiotic G-52[1,5]. Unexpectedly, however, *M. rhodorangea* 5326, like *M. purpurea* 1124, yielded gentamicin C$_{2b}$ (Fig. 1). A 10-liter preparative fermentation was carried out to study the *M. rhodorangea* strain-mediated biotransformation in detail. The results of the examination of the biotransformation products are reported in this paper.

MATERIALS AND METHODS

Strain. *M. rhodorangea* NRRL 5326 was used throughout the investigation.

Shake flask fermentation and isolation of the crude product. Shake flask fermentations were performed in 50 ml of medium (4) in 300-ml Erlenmeyer flasks, each containing 50 mg of sisomicin sulfate and 20 μCi of L-[*methyl*-^{14}C]methionine added at various times. Previously described procedures (3,4) were used for isolation of crude product.

Tank fermentation and isolation of the crude product. A 500-ml amount of the 2-day inoculum was added to 10 liters of fermentation medium in a 14-liter fermentor (New Brunswick Scientific, model MF-114), and the fermentation broth was stirred at 28°C at 350 to 500 rpm with a continuous air flow of 3.5 to 5 liters/min (3). Approximately 20 ml of antifoam (Dow Corning B emulsion) was added automatically during the entire fermentation. One gram of sisomicin sulfate, equivalent to 646 mg of free base, was added 48 h after inoculation; the fermentation broth was harvested after an additional 120-h incubation.

The harvested beer (8.8 liter, pH 6.85) was acidified to pH 2 with concentrated H$_2$SO$_4$, stirred for 1 h, and filtered. The filtrate was neutralized with concentrated NH$_4$OH, and 55.5 g of oxalic acid was added to precipitate calcium. The preparation was refiltered, and the filtrate (pH 1.8) was reneutralized with concentrated NH$_4$OH. A 100-g portion of IRC-50 resin (NH$_4^+$ cycle) was added to the neutralized filtrate, and the mixture (resin plus filtrate) was stirred for 1 h at room temperature. The clean supernatant was poured off, and the resin was washed with distilled water and eluted four times with 1.8, 1.5, 1.0, and 1.0 liter of 2 N NH$_4$OH, respectively. Combined eluates were concentrated, filtered, and lyophilized to yield the crude product.

Column chromatography. An 800-g amount of silica gel (GF 254, type 60 EM) was suspended in a mixture of chloroform–isopropanol–17% ammonia (2:1:1), shaken for 0.5 h, and packed into a column (45 by 7.5 cm). The crude product (1.281 g) was suspended in the same solvent mixture, added to the column, and eluted with the solvent mixture at a flow rate of 0.33 ml/min for the first 274 tubes and a

flow rate of 0.2 ml/min from tube no. 275. A total of 1,965 tubes, containing 10 ml each, was collected. Contents of the tubes giving a positive ninhydrin test were combined, chromatographed, and bioautographed (3).

Radioisotope and tracer work. L-[*methyl-*^{14}C]methionine (New England Nuclear Corp., 40 to 55 mCi/mmol) was used, and previously reported procedures (8) were used for the radioactive tracer work.

Time course study of incorporation of [*methyl-*^{14}C]methionine into gentamicin C$_{2b}$ and antibiotic G-418. L-[*methyl-*^{14}C]methionine (20 μCi) and sisomicin sulfate (50 mg) were added to the fermentation broth of the *M. rhodorangea* 96 h after inoculation. Samples (5 ml) were withdrawn and processed for isolation after incubation of the strain with the isotope and the substrate for various lengths of time (0.25, 6, 24, 48, and 72 h). Radiochromatograms of

the isolated crude products were developed in a descending system of the lower phase of chloroform–methanol–17% ammonia (2:1:1, by volume) and scanned with a Nuclear-Chicago scanner (model 1002). Chromatographic identification of gentamicin C$_{2b}$ and antibiotic G-418 was made, using authentic standards of the antibiotics.

RESULTS

Isolation of crude mixture. From a 10-liter preparative transformation of sisomicin (1 g of sulfate, equivalent to 646 mg of free base), 1.281 g of crude product was isolated by the above method.

Column chromatography. From 1.281 g of the crude product, 30.6 mg of gentamicin C$_{2b}$ (free base, fraction 6 in Table 1 and Fig. 2) and 79.6 mg of sisomicin (free base, fraction 9) were
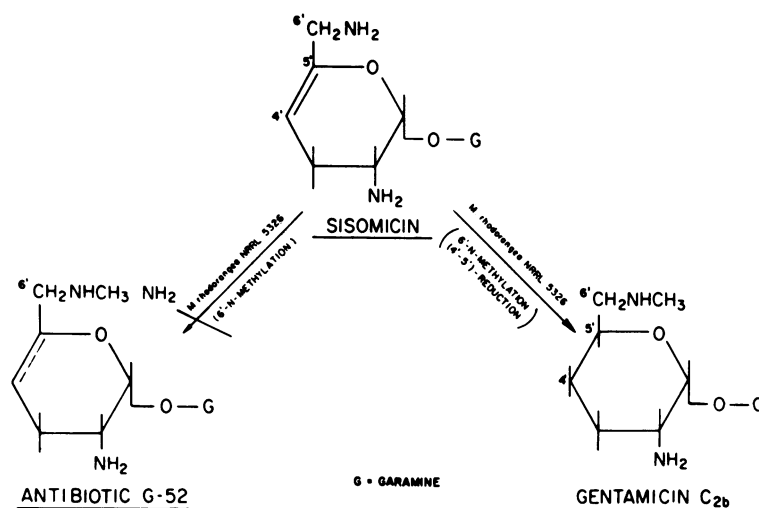

FIG. 1. *Scheme for the bioconversion of sisomicin to gentamicin C$_{2b}$, but not to antibiotic G-52.*

TABLE 1. *Column chromatographic separation of biotransformation products*

Fraction no.	Tube no.	Wt (mg)	Antibiotic
1	1–589	42.3	Inactive metabolite
2	590–690	6.4	Mixture of an unidentified antibiotic (least polar) and gentamicin C$_{2b}$
3	691–720	2.8	Same mixture as fraction 2
4	721–744	2.2	Mixture of the unidentified antibiotic, gentamicin C$_{2b}$, sisomicin, and polar components
5	745–749	0.6	Mixture of gentamicin C$_{2b}$ and polar components
6	750–890	30.5	Gentamicin C$_{2b}$
7	891–1,005	7.2	Mixture of gentamicin C$_{2b}$ and sisomicin
8	1006–1,074	7.0	Sisomicin
9	1075–1,570	79.6	Sisomicin
10	1571–1,965	24.6	Mixture of sisomicin and polar components
11	CMN^a wash	13.1	Same mixture as fraction 10
12	Methanol wash	1,200.0^b	Antibiotic G-418

^{a} Lower phase of chloroform–methanol–concentrated ammonia (2:1:1).
^{b} The 1,200-mg fraction consisted mainly of salts and contained 127 mg of antibiotic G-418.
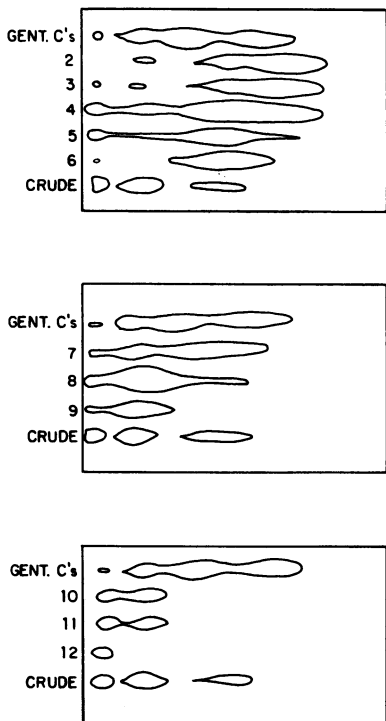
FIG. 2. Bioautograms of chromatographic fractions. From top to bottom: gentamicin C complex, fractions 2, 3, 4, 5, 6, crude; gentamicin C complex, fractions 7, 8, 9, crude; gentamicin C complex, fractions 10, 11, 12 crude.

FIG. 3. Time course for the respective incorporation of label from L-[methyl-^{14}C] methionine into antibiotic G-418 and gentamicin C$_{2b}$.

isolated. Fraction 1 was bioinactive, and fraction 12 was found to contain 127 mg of antibiotic G-418. Fraction 2 (6.4 mg) and fraction 3 (2.8 mg) were shown, respectively, to be a mixture of gentamicin C$_{2b}$ and an unidentified antibiotic, which was chromatographically less polar than gentamicin C$_1$.

Identification of fraction 6. The mass and proton magnetic resonance spectra of a decarbonated sample from fraction 6 (gentamicin C$_{2b}$) were identical to those reported by Daniels and co-workers (2).

Time course study of incorporation of [methyl-^{14}C]methionine. The rates of incorporation of the ^{14}CH$_3$ label into both gentamicin C$_{2b}$ and antibiotic G-418 were highest during the early incubation (0.25 to 6 h) with the organism and leveled off in about 24 h. The ratio of the incorporation of the ^{14}CH$_3$ label into the gentamicin C$_{2b}$ to that incorporated into antibiotic G-418 was approximately 1:2.6 (Fig. 3).

Attempts to monitor the (4′-5′)-reduction process. Gentamicin C$_{2b}$ (the final product) and antibiotic G-52 (a postulated intermediate formed during the transformation of sisomicin
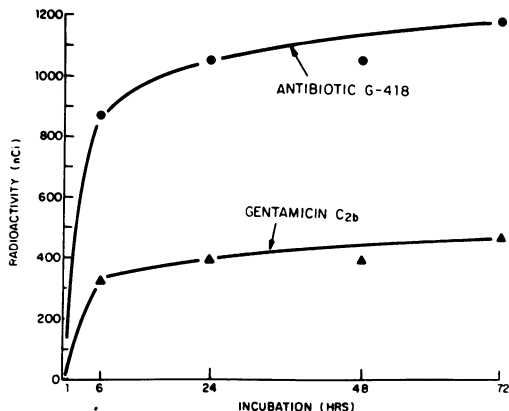
to gentamicin C$_{2b}$), or sisomicin (substrate) and gentamicin C$_{1a}$ (an alternative postulated intermediate), could not be clearly differentiated because of their respective overlap on chromatograms. Consequently, we could not monitor the (4′-5′)-reduction process.

DISCUSSION

The biosynthesis of antibiotic G-418 would require 3″-N-, 4″-C-, and 6′-C-trimethylation of a postulated precursor (3″-N-desmethylgentamicin A$_1$). Daniels and co-workers (P.J.L. Daniels, A. Yehaskel, and J.B. Morton, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 45, 1976) isolated the gentamicin C$_1$ from an L-[methyl-^{13}C]methionine-feeding fermentation. They showed that the carbon enrichments for the 3″-N-, 6′-N-, 4″-C-, and 6′-C-methyl groups were respectively, 2.65, 2.93, 3.07, and 2.26 times greater than in natural abundance and concluded that the carbon atoms of the gentamicin methyl groups are derived from methionine. Therefore, the 6′-N-methylation of sisomicin by the antibiotic G-418-producing strain would have to compete with the 3″-N-, 4″-C-, and 6′-C-methylation for the methyl groups, resulting in a low percentage of incorporation of the ^{14}CH$_3$ label into the gentamicin C$_{2b}$, as seen in the above-mentioned results.

From a preparative biotransformation of 646 mg of sisomicin by *M. rhodorangea* NRRL 5326, we have isolated 30.5 mg (or 5%) of gentamicin C$_{2b}$. We were unsuccessful in following (4′-5′)-reduction, which should have been one of the two enzymatic reactions involved in the biotransformation of sisomicin to gentamicin C$_{2b}$. On the other hand, a time course study of the 6′-N-methylation was possible by chasing

the $^{14}CH_3$ label from the L-[*methyl*-^{14}C]methionine. At present, it is not known whether the gentamicin C_{2b} is formed via (i) the 6'-N-methylation followed by the (4'-5')-reduction of sisomicin, (ii) the reduction followed by the methylation, or (iii) simultaneous reactions of the two.

Based on chromatographic evidence, it is suspected that antibiotic G-418 is a precursor to gentamicin C_2, and gentamicin C_2 is a precursor to gentamicin C_1 (7). Therefore, it is assumed that the antibiotic G-418-producing *M. rhodorangea* is blocked in the 4',5'-dideoxygenation step. The 6'-N-methylation by *M. rhodorangea* seems to be specific to the 4',5'-dideoxy substrates, including gentamicin C_{1a} and sisomicin, and follows the 4',5'-dideoxygenation step in the biosynthesis of the gentamicin antibiotics.

ACKNOWLEDGMENTS

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