SAGAMICIN AND THE RELATED AMINOGLYCOSIDES: FERMENTATION AND BIOSYNTHESIS

I. BIOSYNTHETIC STUDIES WITH THE BLOCKED MUTANTS OF *MICROMONOSPORA SAGAMIENSIS*

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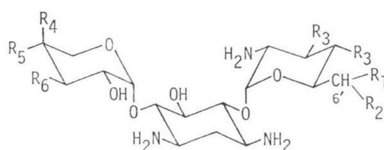
The mutants blocked in the gentamicin C_1 production were derived from a sagamicin producing strain of *Micromonospora sagamiensis*. The intermediates produced by these mutants were isolated and properly identified. Comparing the biotransformation activities in the resting cells of the mutants with those of a DOS idiotroph, KY 11525, the blocked steps in sagamicin and gentamicin biosynthesis were proposed in each mutant. Mutant KY 11564 was found to produce gentamicin C_{2a} (C-6′-epimer of gentamicin C_2) together with gentamicin C_{1a} and sagamicin. KY 11525 transformed gentamicin C_{2a} into C_2 and C_1, whereas KY 11564 lacked the activity. KY 11565 produced gentamicin X_2 and antibiotic G-418, and lacked 6′-amino substitution activities. KY 11566 appeared to be partially blocked in 6′-*N*-methylation activities, and the major products were gentamicin C_{2a} and C_{1a}. From these results, sagamicin biosynthesis in *M. sagamiensis* is discussed.

Sagamicin[1~3] is a new aminoglycoside antibiotic produced by *Micromonospora sagamiensis*. The antibiotic exhibits a potent antibacterial activity against a broad range of Gram-positive and Gram-negative bacteria. Its structure has been characterized as 6′-*N*-methylgentamicin C_{1a}[3] (Fig. 1). The structural differences of sagamicin from gentamicin C occur at the 6′-position (Fig. 1). Several

Fig. 1. The structure of sagamicin and gentamicin components.



	R_1	R_2	R_3	R_4	R_5	R_6
Paromamine	H	OH	OH			
Gentamicin A_2	H	OH	OH	H	OH	OH
Gentamicin A	H	OH	OH	H	OH	$NHCH_3$
Gentamicin X_2	H	OH	OH	OH	CH_3	$NHCH_3$
Antibiotic G-418	CH_3	OH	OH	OH	CH_3	$NHCH_3$
Antibiotic JI-20A	H	NH_2	OH	OH	CH_3	$NHCH_3$
Antibiotic JI-20B	CH_3	NH_2	OH	OH	CH_3	$NHCH_3$
Gentamicin C_{1a}	H	NH_2	H	OH	CH_3	$NHCH_3$
Gentamicin C_2	CH_3	NH_2	H	OH	CH_3	$NHCH_3$
Gentamicin C_1	CH_3	$NHCH_3$	H	OH	CH_3	$NHCH_3$
Sagamicin	H	$NHCH_3$	H	OH	CH_3	$NHCH_3$

minor components which are structuraly related to gentamicin C and sagamicin have been described[4~7]: gentamicins A, A_1, A_2, B and X_2 and antibiotics G-418, JI-20A and JI-20B (Fig. 1). *M. sagamiensis* co-produced a small amount of gentamicin C's and these minor components.

TESTA and TILLEY[8)] demonstrated the bioconversion of the minor gentamicin components into the C complex utilizing a paromamine-producing mutant of *M. purpurea* which was blocked in the production of gentamicin. Based on the chromatographic evidence for the biotransformation of the compounds, they proposed biosynthetic pathways for the gentamicins (Fig. 2): (i) Paromamine to gentamicin A and then to gentamicin X_2. (ii) From gentamicin X_2, the pathway is branched into two; one pathway leading to JI-20A, C_{1a} and then to sagamicin (C_{2b}); the second pathway leading to G-418, JI-20B, C_2 and then to C_1. However, it should be noted that there are limitations in the use of chromatography for identification, and that other intermediates may be involved in their proposed pathways.

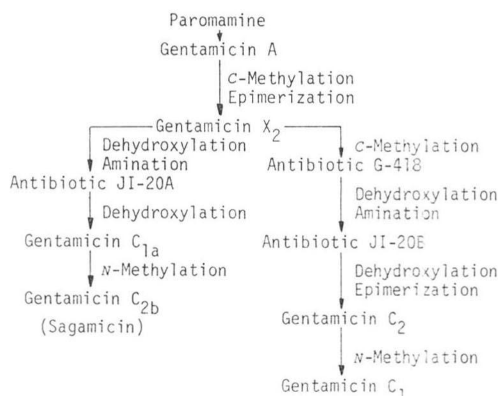


Fig. 2. Proposed biosynthetic pathway for gentamicin (TESTA and TILLEY, 1976[8)]).

In the present investigation, the authors attempted to study the biosynthetic pathways for sagamicin and gentamicin more precisely. A number of blocked mutants were developed from *M. sagamiensis* and the intermediates produced by these mutants were isolated and characterized. Utilizing these blocked mutants and the intermediates isolated, biotransformation experiments were performed. In this paper, the authors report the isolation and characterization of the mutants blocked in the gentamicin C_1 production. Biotransformation activities in the resting cells of the mutants were compared with those of a 2-deoxystreptamine (DOS) idiotroph, and the blocked step in sagamicin and gentamicin biosynthesis is proposed in each mutant. Based on these results, sagamicin biosynthesis in *M. sagamiensis* is discussed.

Materials and Methods

Microorganisms

A sagamicin producing strain of *Micromonospora sagamiensis*, KY 11510, and a DOS idiotroph, KY 11525[9)] were used.

Media

The seed and fermentation media of the following compositions were used. The seed medium; 2% Stabilose K (soluble starch), 0.5% glucose, 0.5% peptone, 0.5% yeast extract, 0.3% meat extract, and 0.2% calcium carbonate (pH 8.0). The fermentation medium; 4% Stabilose K, 1% soy bean meal, 2% Pharmamedia (cotton seed flour), 0.1% corn oil, 0.5% casein, 0.2% phytate (1Ca, 2Mg), 0.015% $FeSO_4 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.025% KH_2PO_4 (pH 8.0).

Culture Condition

First seed culture was developed in a large test tube (2.5×19 cm) containing 10 ml of the seed medium by shaking at 30°C for 3 days. One milliliter of the seed culture was transferred into a large test tube containing the seed medium and incubated for 1 day. Five milliliters of the second seed culture was transferred into a 300-ml flask containing 50 ml of the fermentation medium. The flask was in-

cubated with shaking at 220 rpm at 30°C.

Biotransformation with Resting Cells

The mycelia grown in the fermentation medium for three days were washed three times with 0.1 M tris-HCl buffer (pH 7.5). The washed mycelia were suspended in the same buffer and used for the biotransformation experiments. The reaction mixture, containing 150 mg (dry weight) of the washed cells and 500 μg of each substrate compound in 10 ml of 0.1 M tris-HCl buffer (pH 7.5), was incubated at 30°C for 20 hours with shaking.

Detection of Products

The reaction mixture or fermentation broth was acidified to pH 2.0 with oxalic acid and stirred at 30°C for 1 hour. After centrifugation, the supernatant was neutralized with aqueous ammonia, and applied to Amberlite IRC-50 ion-exchange resin (NH_4^+ form) column. The antibiotic was eluted with 2 N ammonium hydroxide, and the eluate was evaporated to dryness. The dried material was dissolved in distilled water to a desired concentration.

The products were detected by means of carbon[10] and silica gel thin-layer chromatography (TLC) and paper chromatography using the following solvent system: the lower phase of chloroform - methanol - concentrated ammonium hydroxide (1 : 1 : 1, v/v) for silica gel TLC, 2 : 1 : 1 (v/v) (17% ammonium hydroxide) for paper chromatography and 0.5 N hydrochloric acid - methanol (20 : 1, v/v) for carbon TLC. Antibiotic zones on the chromatograms were detected by bioautography against *Bacillus subtilis* ATCC 6633. Silica gel TLC was also visualized following RYDON-SMITH reaction.

The amount of the individual product was determined by *in-situ* fluorometric measurements of the 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole derivatives formed on silica gel TLC plates.[11]

Isolation of Fermentation Products

The fermentation broth was acidified to pH 2.0 with sulfuric acid and stirred for 1 hour at 60°C. After filtration, the filtrate was applied to Diaion HPK-25 resin (NH_4^+) column and eluted with 2 N ammonium hydroxide. The eluate was neutralized with 0.5 N hydrochloric acid and applied to Amberlite CG-50 resin (NH_4^+) column. The resin was washed with distilled water and eluted with a linear gradient of 0.05 to 0.5 N ammonium hydroxide. Fractions were monitored using silica gel TLC or paper chromatography. Similar fractions were combined, evaporated under reduced pressure to a small volume and lyophilized to yield crude preparations. Each preparation was further chromatographed on silica gel (Wako gel C-200) column. Antibiotic G-418 and gentamicin X_2 were eluted with *n*-butanol - ethanol - conc. ammonium hydroxide (8 : 10 : 7, v/v), and sagamicin and gentamicins C_{1a}, C_2 and C_1 with the lower phase of chloroform - methanol - conc. ammonium hydroxide (3 : 1 : 1, v/v). Carbon column chromatography was also used for the separation of gentamicin C_2 from C_{2a}[12].

The antibiotics isolated were identified from the biological properties, chromatographic behavior, and mass and proton NMR spectra. Those properties of the antibiotics (gentamicin X_2 and antibiotic G-418 isolated from KY 11565, gentamicin C_{1a}, C_{2a} and sagamicin from KY 11564, and gentamicin C_{2a} and C_{1a} from KT 11566) were each identical to the authentic samples[3~5,7,12,13].

Materials

Gentamicins A, A_2 and X_2, and antibiotics JI-20A, JI-20B and G-418 used for the biotransformation experiments were isolated from the cultured broth of *M. sagamiensis* mutant KY 11529 by chromatographic procedures[14]. Sagamicin and gentamicins C_{1a}, C_2 and C_1 used for the biotransformation experiments were isolated from a culture broth of KY 11510 by the method described above. Authentic gentamicins were kindly supplied from Dr. G. H. WAGMAN of the Schering Corporation.

Results

Biotransformation of Gentamicin Components by the Resting Cells of a DOS Idiotroph KY 11525

Biotransformation of gentamicin components was examined using the resting cells of a DOS idiotrophic mutant, KY 11525. Gentamicin C_{1a} was effectively converted to sagamicin. The formation of

Fig. 3. a) Effect of mycelium concentration on sagamicin formation from gentamicin C_{1a}.

Reaction mixture containing 500 μg of gentamicin C_{1a} sulfate and the increasing concentration of mycelia (mg dry weight/ml) in 10 ml of 0.1 M tris-HCl buffer (pH 7.5) was incubated at 30°C for 20 hours.

b) Effect of gentamicin C_{1a} concentration on sagamicin formation.

Reaction mixture containing 113 mg of washed mycelia and the increasing amount of gentamicin C_{1a} sulfate in 10 ml of 0.1 M tris-HCl buffer (pH 7.5) was incubated at 30°C for 20 hours.

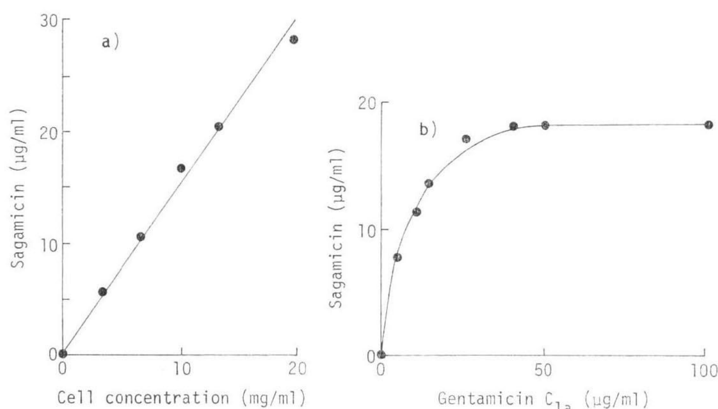


Fig. 4. Effect of *S*-adenosyl-L-methionine on sagamicin formation from gentamicin C_{1a} in *M. sagamiensis*.

The reaction mixture contained 250 μg gentamicin C_{1a} sulfate, 42 mg washed mycelia, and the increasing amounts of *S*-adenosyl-L-methionine in 5 ml of 0.1 M tris-HCl buffer (pH 7.5). The reaction was carried out at 30°C for 20 hours.

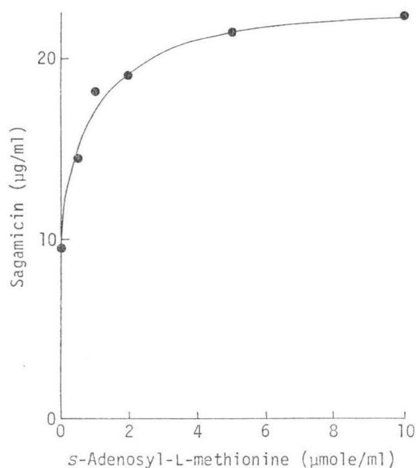


Table 1. Biotransformation of gentamicin components in the resting cells of a DOS idiotroph of *M. sagamiensis*, KY 11525.

Substrate	Biotransformation products
DOS	Gentamicin C_{1a}, C_2, C_1 and sagamicin
Paromamine	Gentamicin C_{1a}, C_2, C_1 and sagamicin
Gentamicin A	Gentamicin C_{1a}, C_2, C_1 and sagamicin
Gentamicin X_2	Gentamicin C_{1a}, C_2, C_1 and sagamicin
Antibiotic G-418	Gentamicin C_2 and C_1
Antibiotic JI-20A	Gentamicin C_{1a} and sagamicin
Antibiotic JI-20B	Gentamicin C_2 and C_1
Gentamicin C_{1a}	Sagamicin
Gentamicin C_2	Gentamicin C_1
Gentamicin C_1	—
Sagamicin	—

The reaction mixture containing 1000 μg (DOS, paromamine or gentamicin A) or 500 μg (other compounds) of substrate and 113 mg of washed mycelia in 10 ml of 0.1 M tris-HCl buffer (pH 7.5) was incubated at 30°C for 20 hours.

sagamicin was dependent on the concentration of the cells (Fig. 3-a) and gentamicin C_{1a} (Fig. 3-b). Sagamicin formation from gentamicin C_{1a} was markedly stimulated by *S*-adenosyl-L-methionine (Fig. 4). Addition of cobalt chloride (100 and 1000 μg/liter) did not affect sagamicin formation.

The results of the biotransformation of various gentamicin components in this system are summarized in Table 1. DOS, paromamine, gentamicin A or X_2 was transformed to C_{1a}, C_2, C_1 and sagamicin; G-418 or JI-20B to C_2 and C_1; C_2 to C_1; JI-20A to C_{1a} and sagamicin; C_{1a} to sagamicin. The biotransformation activities from the presumed early intermediates (DOS, paromamine, gentamicin A or X_2) to gentamicin C_{1a} and sagamicin were higher than the activities to gentamicins C_2 and C_1. Gentamicin C_1 and sagamicin were not transformed to any detectable antibiotics. These results were in agreement with those from the growing cells of *M. purpurea*[8] although the transformation activities from the early intermediates in *M. purpurea* were higher to gentamicins C_2 and C_1 than to sagamicin.

The Mutants Blocked in Gentamicin C_1 Pathway

M. sagamiensis KY 11510 co-produced gentamicin C_1 with sagamicin in the fermentation. When KY 11510 was cultivated in the fermentation medium supplemented with cobalt chloride (1 mg/liter), the amount of gentamicin C_1 production exceeded that of sagamicin[15]. The mutants which produced no gentamicin C_1 in the cobalt supplemented medium were selected from NTG-treated population of KY 11510[15]. A variety of mutants blocked in gentamicin C_1 pathway were isolated. Fermentation products by these mutants are shown in Table 2. These products were isolated by the method described in "Materials and Methods", and identified on the basis of the chromatographic behavior, biological properties, and mass and proton NMR spectral analysis. Mutant KY 11538 produced sagamicin, but did not produce gentamicins C_1, C_2 and C_{2a} even in the presence of cobalt[15]. KY 11565 produced gentamicin X_2 and antibiotic G-418. Addition of cobalt ion was found to have a significant effect upon the titer of both antibiotics without affecting the growth of the mutant. In the absence of cobalt ion, KY 11565 produced 240 μg/ml of gentamicin X_2 and 122 μg/ml of G-418. Addition of 100 μg/liter of cobalt chloride resulted in a 2-fold decrease of gentamicin X_2 and a 1.6-fold increase of G-418 (Fig. 5). KY 11564 produced sagamicin, gentamicins C_{1a} and C_{2a} (6'-*C*-epimer of gentamicin C_2) but did not produce gentamicin C_2 or C_1. Gentamicin C_{2a} was clearly differentiated from gentamicin C_2 by carbon TLC[12]. KY 11566 produced gentamicins C_{1a} and C_{2a}. A small amount (below 10% of C_{2a}) of gentamicins C_2, C_1 and sagamicin were also detected. Cobalt ion affected the production of these antibiotics (Table 3).

Table 2. The fermentation products of the blocked mutants of *M. sagamiensis*.

Strain No.	Major fermentation products
KY 11510 (parent)	Sagamicin, gentamicin C_1
KY 11565	Gentamicin X_2, antibiotic G-418
KY 11564	Gentamicins C_{1a} and C_{2a}, sagamicin
KY 11566	Gentamicins C_{2a} and C_{1a}
KY 11538*	Sagamicin

* See reference 15.

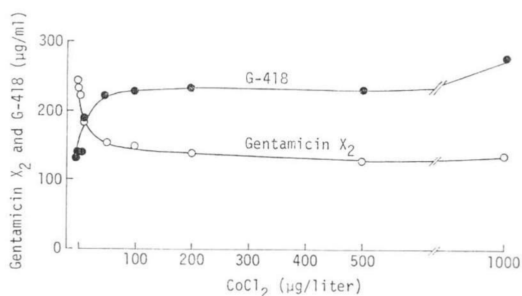


Fig. 5. Effect of cobalt chloride on the production of gentamicin X_2 and G-418 in KY 11565.

KY 11565 was cultivated in the fermentation medium at 30°C for 8 days.

Table 3. Production of the antibiotics by *M. sagamiensis* mutants KY 11564 and KY 11566.

Strain No.	Production (μg/ml)							
	Sagamicin		Gentamicin C_1		Gentamicin C_{1a}		Gentamicin C_{2a}	
	$CoCl_2^+$	$CoCl_2^-$	$CoCl_2^+$	$CoCl_2^-$	$CoCl_2^+$	$CoCl_2^-$	$CoCl_2^+$	$CoCl_2^-$
KY 11566	—	—	—	—	83	78	186	30
KY 11564	116	223	—	—	48	41	267	73

The mutants were cultivated in the fermentation medium in the presence ($CoCl_2^+$) or in the absence ($CoCl_2^-$) of 1 mg/liter of cobalt chloride at 30°C for 8 days.

Biosynthetic Relationship between Gentamicins C_{2a} and C_2

Although gentamicin C_{2a} was suggested, from its structure, as a biosynthetic precursor of gentamicin C_1[8], no evidence for biotransformation of the antibiotic to gentamicin C_1 have been reported. Using carbon TLC to differentiate gentamicin C_{2a} from C_2, biotransformation of these antibiotics by KY 11525 could be demonstrated. As shown in Table 4, gentamicin C_{2a} was transformed to C_2 and C_1, and gentamicin C_2 to C_{2a} and C_1. The transformation activity from C_{2a} to C_2 was much higher than that from C_2 to C_{2a}. Both activities were completely abolished by heating the mycelium at 100°C for 5 minutes. Antibiotic JI-20B was transformed into gentamicins C_{2a}, C_2 and C_1.

Biotransformation with the Resting Cells of the Blocked Mutants

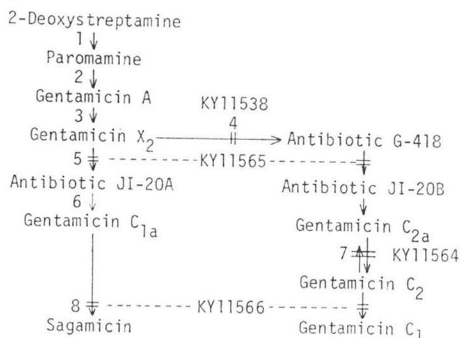
The biotransformation of gentamicin components was performed with the resting cells of the blocked mutants (Table 5). KY 11565, the producer of gentamicin X_2 and G-418, transformed JI-20A to gentamicin C_{1a} and sagamicin, JI-20B to gentamicins C_2 and C_1; gentamicin C_{1a} to sagamicin; gentamicin C_{2a} to C_1; and gentamicin C_2 to C_1. But the mutant lacked the activity to transform gentamicin A, X_2 or G-418 to gentamicin C's or sagamicin. The results suggest that the blockage in amino substitution activities at C-6′ of both gentamicin X_2 and G-418 (Step 5 in Fig. 6) in KY 11565 resulted in the

Table 4. Biotransformation of gentamicins C_{2a} and C_2 by *M. sagamiensis* KY 11525.

Mycelia	Substrate	Biotransformation products		
		C_{2a}	C_2	C_1
Washed mycelia	C_{2a}		++	+
	C_2	+		+
	None	—	—	—
Boiled mycelia	C_{2a}		—	—
	C_2	—		—
	None	—	—	—

Reaction mixture; 144 mg washed mycelia of KY 11525 and 150 μg substrate in 5 ml of 0.1 M tris-HCl buffer (pH 7.5). Incubated at 30°C for 20 hours.

Fig. 6. The biosynthetic pathway of sagamicin and gentamicin and blocked steps in the mutants of *M. sagamiensis*.



1. Addition of D-glucosamine. 2. Addition of D-xylose, amino substitution and *N*-methylation at C-3″. 3. *C*-Methylation, with inversion of configuration, at C-4″. 4. *C*-Methylation at C-6′ with inversion of configuration. 5. Amino substitution at C-6′. 6. 3′,4′-Dehydroxylation. 7. Epimerization at C-6′. 8. *N*-Methylation at C-6′.

Table 5. Biotransformation with the mutants blocked in the gentamicin C_1 pathway.

Substrate	KY 11565	KT 11564	KY 11566
None	ND	ND	ND
Gentamicin A	ND	NT	NT
Gentamicin X_2	ND	NT	NT
Antibiotic G-418	ND	NT	NT
Antibiotic JI-20A	Gentamicin C_{1a} Sagamicin	Sagamicin	Gentamicin C_{1a}
Antibiotic JI-20B	Gentamicin C_2 Gentamicin C_1	Gentamicin C_{2a}	Gentamicin C_{2a}
Gentamicin C_{1a}	Sagamicin	Sagamicin	(Sagamicin)
Gentamicin C_{2a}	Gentamicin C_1	ND	NT
Gentamicin C_2	Gentamicin C_1	Gentamicin C_1	(Gentamicin C_1)

The reaction mixture contained 250 μg of the substrate compound, 78 mg washed mycelia in 5 ml of 0.1 M tris-HCl buffer (pH 7.5). The reaction was carried out at 30°C for 20 hours.
ND; not detected, NT; not tested.

production of gentamicin X_2 and G-418. KY 11564, which produced sagamicin, gentamicins C_{1a} and C_{2a}, transformed JI-20A or gentamicin C_{1a} to sagamicin, JI-20B to C_{2a}, and C_2 to C_1. However, the mutant did not transform JI-20B or C_{2a} to C_2 and C_1. Based on the results, KY 11564 is apparently blocked in the C-6′ epimerization activity (Step 7 in Fig. 6). Moreover, the results supported the transformation sequence, $C_{2a} \rightarrow C_2 \rightarrow C_1$. KY 11566 hardly transformed JI-20A to sagamicin, or JI-20B to gentamicin C_1. But it possessed a weak activity for transformation of gentamicin C_{1a} to sagamicin, and gentamicin C_2 to C_1. From these results, KY 11566 appears to be partially blocked in 6′-*N*-methylation activity (Step 8 in Fig. 6).

Discussion

Based on the results of the biotransformations and the intermediates produced by the blocked mutants, sagamicin biosynthetic pathway in *M. sagamiensis* is proposed as shown in Fig. 6. Four kinds of blocked mutants including those blocked in the 6′-*C*-methylation step[15] were obtained by selecting the non-gentamicin C_1-producing mutants. Two mutants, KY 11565 and KY 11566, were blocked in sagamicin production as well as in gentamicin C_1 production. KY 11565 appeared to have a blockage in 6′-amino substitution of gentamicin X_2 and G-418, and KY 11566 in 6′-*N*-methylation of gentamicins C_{1a} and C_2. The results suggest that the 6′-amino substitution and the 6′-*N*-methylation, involved in both sagamicin and gentamicin C_1 synthesis are each catalized by a single enzyme. Similarly, a single enzyme may be involved in the 3′,4′-dehydroxylation (Step 6), but no mutants blocked in the step have been isolated in *M. sagamiensis*.

Involvement of gentamicin C_{2a} in the gentamicin C_1 pathway was demonstrated with KY 11564, which is blocked in the 6′-epimerization of gentamicin C_{2a}. The parent strain, KY 11510, produced both gentamicin C_{2a} and C_2 in equal amounts. KY 11564 produced gentamicin C_{2a}, sagamicin and gentamicin C_{1a}. The mutant possessed no activity to transform gentamicin C_{2a} to C_2. Utilizing a DOS idiotroph, KY 11525, it was demonstrated that the 6′-*C* epimerization step was reversible.

In our biotransformation experiments, the presumed early intermediates were not isolated: paromamine, gentamicin A, X_2, JI-20A, G-418, or JI-20B in the biotransformation of DOS; gentamicin A, X_2 JI-20A, G-418 or JI-20B in the biotransformation of paromamine; gentamicin X_2, JI-20A, G-418 or JI-20B in the biotransformation of gentamicin A; or JI-20A, G-418 or JI-20B in the biotransformation of gentamicin X_2. However, the studies with KY 11565 and KY 11538[15] strongly support that gentamicin X_2 and G-418 is the early precursors. Further investigation may be required to verify other

intermediates in the early steps of the proposed pathway (Fig. 6).

Cobalt ion was shown to have a significant effect on the production of gentamicin X_2 and G-418 in KY 11565. TESTA and TILLEY[16] found that the *C*-methylation at the 6′-position and the 4′′-position (step 3 and 4 in Fig. 6) depended on cobalt ion in *M. purpurea*. The increase in the production of G-418, accompanying the decrease in gentamicin X_2, in KY 11565 may be due to the stimulation of one of the cobalt-dependent steps (Step 4 in Fig. 6). Sagamicin production in the mutants blocked in step 4 was significantly stimulated by the addition of cobalt[15]. Activation of 4′′-*C*-methylation by cobalt may lead to the stimulation of sagamicin production in the mutants. Cobalt ion did not affect the conversion activity from gentamicin C_{1a} to sagamicin. These results strongly support that the cobalt-dependent steps involved in the *C*-methylations, but not in the *N*-methylation.

S-Adenosyl-L-methionine significantly stimulated the conversion from gentamicin C_{1a} to sagamicin in the resting cells of KY 11525. DEGUCHI *et al.*[17] reported that *S*-adenosyl-L-methionine-[methyl-^{14}C] was incorporated into sagamicin at a low efficiency (4.1 %). It may be suggested that methyl donor for the 6′-*N*-methylation is *S*-adenosyl-L-methionine.

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