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Biotransformation of Sisomicin and Verdamicin by *Micromonospora sagamiensis*†

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The resting cells of a 2-deoxystreptamine idiotrophic mutant of *Micromonospora sagamiensis* were found to transform sisomicin into gentamicin C_{1a} and sagamicin. The biotransformation products were isolated by a combination of ion exchange and carbon column chromatographic procedures, and properly identified. Antibiotic G-52 (6′-*N*-methylsisomicin) was not detected in the transformation products. Gentamicin C_{1a} was formed at an early stage of the biotransformation, followed by sagamicin formation. The (4′,5′)-reduction of sisomicin may occur first, followed by 6′-*N*-methylation. By use of a similar procedure, it was demonstrated that verdamicin was transformed into gentamicin C_{2a} (the 6′-C epimer of gentamicin C_2), C_2, and then C_1. Carbon TLC clearly separated gentamicin C_{2a}, C_2, and verdamicin. In this biotransformation, the (4′,5′)-reduction of verdamicin occurred first, followed by 6′-C-epimerization, and then 6′-*N*-methylation.

In contrast with *M. sagamiensis*, *M. zionensis* NRRL5466 and *M. inyoensis* NRRL3292 did not possess any detectable activity of the (4′,5′)-reduction of sisomicin or verdamicin. Alternatively, NRRL5466 transformed sisomicin into antibiotic G-52, and verdamicin into a new antibiotic, VF3-1. The antibiotic VF3-1 was suggested as 6′-*N*-methylverdamicin by chromatographic and mass spectrum data. The implications of these findings were discussed in relation to sagamicin biosynthesis in *M. sagamiensis*.

In a previous report,[1] it was demonstrated that resting cells of a 2-deoxystreptamine (DOS) idiotrophic mutant of *Micromonospora sagamiensis* transformed gentamicin minor components into sagamicin and gentamicin C. During these studies, transformation of sisomicin-group antibiotics was examined. As shown in Fig. 1, the structure of sisomicin-group antibiotics, sisomicin, verdamicin and antibiotic G-52, was characterized as 4′,5′-dehydro derivatives of gentamicin C_{1a}, C_2, and sagamicin, respectively. These antibiotics were found in the culture broths of *M. inyoensis*,[2] *M. grisea*,[3] and *M. zionensis*,[4] and were thought to be produced *via* a different biosynthetic route from that of sagamicin and gentamicins in *M. sagamiensis* and *M. purpurea*. The authors found that sisomicin and verdamicin were transformed into sagamicin and/or gentamicin Cs by the DOS idiotroph of *M. sagamiensis*. Biotransformation products were isolated and properly identified. Based on the results, the biotransformation sequences were proposed.

In contrast with *M. sagamiensis*, an antibiotic G-52 producing *M. zionensis* transformed sisomicin into G-52 and verdamicin into a new antibiotic, VF3-1.

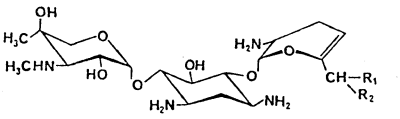
Sagamicin biosynthesis was discussed on the basis of these findings.

MATERIALS AND METHODS

Microorganisms. *Micromonospora sagamiensis* KY11525 (a DOS idiotroph derived from a sagamicin producer, KY11505),[5] *M. inyoensis* NRRL3292, and *M. zionensis* NRRL5466 were used.

Media and culture condition. The compositions of the seed and fermentation media and culture condition were described in a previous paper.[1]

† Sagamicin and the Related Aminoglycosides: Fermentation and Biosynthesis. Part II. For Part I, see ref. 1.



	R_1	R_2
SISOMICIN	NH_2	H
ANTIBIOTIC G-52	$NHCH_3$	H
VERDAMICIN	NH_2	CH_3

FIG. 1. The Structure of Sisomicin-group Antibiotics.

Biotransformation with resting cells. 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. A reaction mixture, containing 150 mg (dry weight) of washed cells and 500μg of each substrate compound in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5), was incubated at 30°C for 20 hr with shaking.

Detection of biotransformation products. The reaction mixture was acidified to pH 2 with oxalic acid and stirred at 30°C for 1 hr. 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.

Biotransformation products were detected by means of carbon-[7] and silica gel-thin layer chromatography (TLC) and paper chromatography using the following solvent systems: 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*, or by Rydon-Smith reagent.

Isolation of biotransformation products. The reaction mixture was acidified to pH 2 with sulfuric acid and stirred at 60°C for 1 hr. 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 HCl 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, as shown in Tables I and II. The crude preparations were further chromatographed on charcoal (Wako) column. Elution was performed with 0.5 N HCl.

Materials. Sisomicin and verdamicin were isolated from a culture broth of *M. grisea* by the chromatographic procedure.[1]

RESULTS

Biotransformation of sisomicin and verdamicin

In the course of the biotransformation studies with the resting cells of *M. sagamiensis* KY11525, sisomicin and verdamicin were found to be transformed to the antibiotics which showed similar *Rf* values to sagamicin and gentamicin C_1, respectively, on a paper chromatogram (Fig. 2). Differentiation of an individual component from the mixture of sisomicin- and gentamicin-group antibiotics is very difficult by silica gel TLC or paper chromatography. Recently, Lee *et al.*[6] reported that G-418-producing *M. rhodorangea* transformed sisomicin to sagamicin (gentamicin C_{2b}), but they failed to elucidate the biotransformation sequence. Postulated intermediates (gentamicin C_{1a} and antibiotic G-52) could not be differentiated from sisomicin and sagamicin

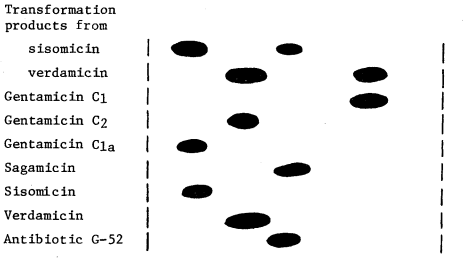


FIG. 2. Paper Chromatogram of the Transformation Products from Verdamicin and Sisomicin in *M. sagamiensis* KY 11525.

Filer paper: Toyo No. 51 (1 × 40 cm, Toyo Roshi Co., Ltd.)

Solvent system: a lower phase of $CHCl_3$–CH_3OH–17% NH_4OH (2 : 1 : 1, v/v). Developed at 28°C for 18 hr.

Bioautographed on *B. subtilis*.

by chromatography. Since carbon TLC was reported to be effective for the differentiation of these antibiotics,[7] the authors attempted to examine the biotransformation of sisomicin and verdamicin by using carbon TLC.

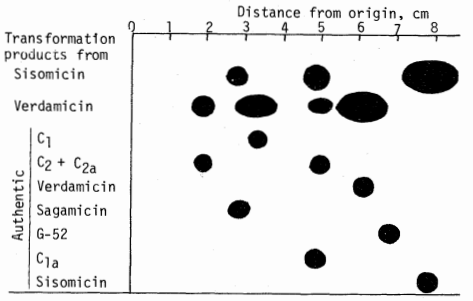


FIG. 3. Carbon Thin-layer Chromatogram of the Biotransformation Products from Sisomicin and Verdamicin in *M. sagamiensis* KY 11525.

Developed with 0.5 N HCl–CH_3OH (20:1, v/v). The developed plate was covered by a sheet of filter paper (20 × 20, Toyo Filter Paper No. 51) wetted by 50% aqueous methanol. After pressing the sheet tightly for 15 min on the carbon surface, the filter paper was removed and exposed to ammonia gas for 5 min. The neutralized filter paper was bioautographed on an agar plate seeded with *B. subtilis*.

As shown in Fig. 3, antibiotic zones corresponding to sagamicin and gentamicin C_{1a} were detected on a carbon thin-layer chromatogram of the biotransformation mixture from sisomicin. Antibiotic G-52, which showed a similar *Rf* value to sagamicin on a paper chromatogram, was not detected by the carbon TLC. The biotransformation products of verdamicin are also shown in Fig. 3. Three antibiotic zones other than the residual verdamicin zone were detected on the carbon TLC. They showed a similar *Rf* value to gentamicin C_2, C_{2a} (the C-6′ epimer of C_2[1,8]) and C_1, respectively.

Isolation and identification of biotransformation products from sisomicin

Biotransformation products were isolated from a 30 liter preparative transformation of sisomicin (1.5 g of sulfate, equivalent to 969 mg free base). Three fractions each containing a crude antibiotic mixture were obtained after

TABLE I. COLUMN CHROMATOGRAPHIC SEPARATION OF BIOTRANSFORMATION PRODUCTS FROM SISOMICIN IN *M. sagamiensis* KY 11525

The washed mycelia (3000 g, wet weight) of *M. sagamiensis* KY 11525 and 750 mg of sisomicin sulfate in 15 liter of 0.1 M Tris-HCl buffer (pH 7.5) were incubated in 30 liter jar fermentor at 400 rpm at 30°C for 3 hr. The biotransformation products were isolated from the reaction mixture (30 liter) as described in MATERIALS AND METHODS.

IRC-50 (NH_4^+) resin		Carbon column chromatography		
Fraction No.	Main component	Fraction No.	wt. mg	Antibiotic
1	Sisomicin	1-1	82.4	Sisomicin
	Sagamicin	1-2	8.6	Sisomicin, sagamicin
		1-3	2.0	Sagamicin
2	Sisomicin	2-1	55.0	Sisomicin
	Sagamicin	2-2	10.9	Sagamicin
		2-3	40.0	Sagamicin An unidentified antibiotic
3	Gentamicin C_{1a}	3-1	38.3	Gentamicin C_{1a}
		3-2	10.0	Gentamicin C_{1a} An unidentified antibiotic

TABLE II. COLUMN CHROMATOGRAPHIC SEPARATION OF BIOTRANSFORMATION PRODUCTS FROM VERDAMICIN IN *M. sagamiensis* KY 11525

The washed mycelia (3000 g, wet weight) of *M. sagamiensis* KY 11525 and 495 mg of verdamicin sulfate in 15 liter of 0.1 M Tris-HCl buffer (pH 7.5) were incubated in 30 liter jar fermentor at 300 rpm at 30°C for 20 hr. The biotransformation products were isolated from the reaction mixture (30 liter) as described in MATERIALS AND METHODS.

IRC-50 (NH_4^+) resin		Carbon column chromatography		
Fraction No.	Main component	Fraction No.	wt. mg	Antibiotic
1	Gentamicins C_2, C_1 and C_{1a}	1-1	35.5	An unidentified antibiotic Gentamicin C_2
		1-2	46.2	Gentamicin C_2
		1-3	45.0	Gentamicins C_2 and C_1
		1-4	50.0	Gentamicin C_1
		1-5	65.0	Gentamicins C_1 and C_{2a} An unidentified antibiotic
		1-6	34.1	Gentamicin C_{2a}
2	Gentamicin C_1	2-1	186.0	Verdamicin
	Verdamicin	2-2	10.0	Gentamicin C_1

IRC-50 resin column chromatography (see MATERIALS AND METHODS). Each fraction was further applied to carbon column chromatography. As shown in Table I, 12.9 mg of sagamicin (free base, fractions 1-3 and 2-2), 38.3 mg of gentamicin C_{1a} (free base, fraction 3-1), and 137.4 mg of sisomicin (free base fraction 1-1 and 2-1) were isolated. Fraction 1-2 (8.6 mg) was a mixture of sisomicin and sagamicin. Fraction 2-3 (40 mg) was a mixture of sagamicin and an unidentified antibiotic, which was chromatographically more polar than gentamicin C_{1a}. Fraction 3-2 (10.0 mg) was a mixture of gentamicin C_{1a} and an unidentified antibiotic. No Antibiotic G-52 was detected in the reaction mixture. The mass and proton magnetic resonance spectra of fractions 1-3 and 2-2 (sagamicin), fraction 3-1 (gentamicin C_{1a}), and fractions 1-1 and 2-1 (sisomicin) were identical to those reported by Egan *et al.*[9] and Reimann *et al.*[10]

Isolation and identification of biotransformation products from verdamicin

By use of a procedure similar to that described above, biotransformation products of verdamicin were isolated from a 30 liter reaction mixture. Two fractions obtained by IRC-50 resin column chromatography were further applied to carbon column chromatography (Table II). Consequently, 46.2 mg of gentamicin C_2 (free base, fraction 1-2), 34.1 mg of gentamicin C_{2a} (free base, fraction 1-6), 60 mg of gentamicin C_1 (free base, fractions 1-4 and 2-2), and 186 mg of verdamicin (free base, fraction 2-1) were isolated. Fraction 1-1 (35.5 mg) was found to be a mixture of gentamicin C_2 and an unidentified antibiotic which showed a chromatographic behavior similar to verdamicin but was not verdamicin from other properties. Fraction 1-3 (45.0 mg) was a mixture of gentamicin C_2 and C_1 and fraction 1-5 (65.0 mg) was a mixture of C_1, C_{2a} and an unidentified antibiotic. The mass and proton magnetic resonance spectra of fractions 1-4 and 2-2 (gentamicin C_1), fraction 1-2 (gentamicin C_2) and fraction 1-6 (gentamicin C_{2a}) were identical to those reported by Egan *et al.*[9] and Daniels *et al.*[8] Gentamicin C_2 and C_{2a} were found to be clearly separated by

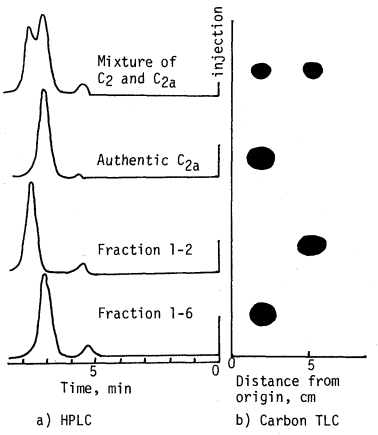


FIG. 4. Comparison of Gentamicin C_2 and C_{2a} by Carbon TLC and HPLC.

a) HPLC:
Column: Yanapak ODS
Solvent: 0.02 M $C_5H_{11}SO_2Na$, 0.2 M Na_2SO_4, 0.1% CH_3COOH and 1% CH_3OH.
Fluorescence detection

b) Carbon TLC
Solvent: 0.5 N HCl–CH_3OH (20 : 1, v/v).
With other conditions, see the legend of Fig. 3.

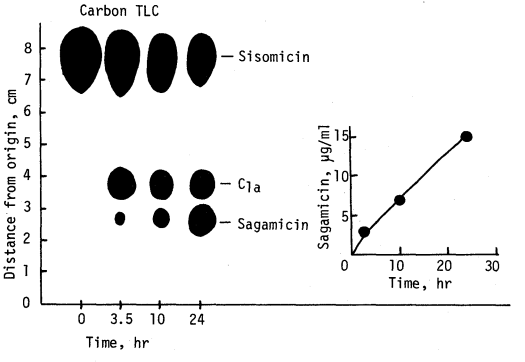


FIG. 5. Time Course of Biotransformation from Sisomicin by *M. sagamiensis* KY 11525.

Reaction mixture: 150 mg of washed mycelia of KY 11525 and 500 μg of sisomicin sulfate in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5). Incubated at 30°C by shaking. Biotransformation products were detected by carbon TLC using a solvent system, 0.5 N HCl–methanol (20 : 1, v/v). The detection was performed with bioautography against *B. subtilis*. With other conditions, see the legend of Fig. 3.

carbon TLC and HPLC. In these systems, fraction 1-2 and fraction 1-6 showed similar *Rf* value and retention time to the authentic gentamicin C_2 and C_{2a}, respectively (Fig. 4).

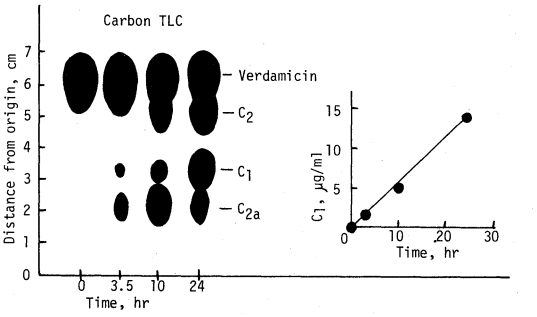


FIG. 6. Time Course of Biotransformation from Verdamicin by *M. sagamiensis* KY 11525.

Reaction mixture: 150 mg of washed mycelia of KY 11525 and 500 μg of verdamicin sulfate in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5). Incubated at 30°C by shaking. With other conditions, see the legend of Fig. 5.

TABLE III. BIOTRANSFORMATION OF SISOMICIN AND VERDAMICIN BY *M. zionensis* NRRL 5466 AND *M. inyoensis* NRRL 3292

Reaction mixture: 112 mg of washed mycelia of NRRL 5466 or NRRL 3292 and 500 μg of substrate in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5). Incubated at 30°C for 18 hr.

Organism	Substrate	Biotransformation product
M. zionensis NRRL 5466	Sisomicin	Antibiotic G-52
	Antibiotic G-52	—
	Verdamicin	Antibiotic VF 3-1
M. inyoensis NRRL 3292	Sisomicin	—
	Antibiotic G-52	—
	Verdamicin	—

Time course of biotransformation

Time course of biotransformation of sisomicin was examined with the resting cells of KY11525. Gentamicin C_{1a} appeared to be formed at an early stage of the biotransformation and reached its maximum level within 3.5 hr. Sagamicin formation occurred after C_{1a} formation and increased linearly up to 24 hr (Fig. 5).

Time course of biotransformation of verdamicin is shown in Fig. 6. Formation of gentamicin C_{2a} occurred first, followed by gentamicin C_2 and C_1.

Mutant KY11565, which appeared to be

blocked in 6′-epimerase,[1] transformed verdamicin to gentamicin C_{2a} but not to C_2 or C_1.

Biotransformation of sisomicin and verdamicin by M. inyoensis and M. zionensis

A sisomicin producer, *M. inyoensis* NRRL3292 and a G-52 producer, *M. zionensis* NRRL5466 were found to have no activity to transform sisomicin or verdamicin to gentamicins or sagamicin. Alternatively, *M. zionensis* NRRL5466 transformed sisomicin and verdamicin into G-52 and an unidentified antibiotic, VF3-1, respectively (Table III). Formation of G-52 and VF3-1 was remarkably stimulated by *S*-adenosylmethionine. *M. inyoensis* NRRL3292 appeared to have no activity to transform sisomicin, verdamicin and G-52.

Isolation and properties of VF3-1

An attempt was made to isolate VF3-1 from a 6 liter preparative transformation of verdamicin (200 mg of sulfate). After incubation at 30°C for 21 hr, the reaction mixture was acidified to pH 2.0 with sulfuric acid and stirred at 80°C for 10 min. 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 HCl and applied to Amberlite CG-50 resin (NH_4^+) column. The resin was washed with distilled water and eluted with 0.1 N ammonium hydroxide. Fractions containing VF3-1 were collected and lyophilized to yield crude VF3-1. The crude VF3-1 was chromatographed on Wako-gel C-200 column. Elution was performed with the lower phase of chloroform–methanol–concentrated ammonium hydroxide (2 : 1 : 1). Fractions containing VF3-1 were combined and the pooled fraction were lyophilized to yield 2.8 mg of VF3-1.

VF3-1 was compared with a variety of antibiotics including gentamicins, sisomicin, and G-52, by bioautography of paper and thin layer chromatograms. The compound was clearly differentiated from other related antibiotics (Tables IV and V). After development with the lower phase of a chloroform–methanol–concentrated ammonium hydroxide (1 : 1 : 1) on silica gel TLC, the *Rf* of VF3-1 was located between gentamicin C_1 and sagamicin (Table V). The color produced by heating at 100°C after treatment with 0.25% ninhydrin in pyridine–acetone was reddish brown during the early stage (3 min), but finally turned to purple. The mass spectrum showed an M^+ peak at *m/z* 475, and major fragments, *m/z* 362, 322, 304, 284, 203, 191, 163, 160, and 155. These data suggested VF3-1 as 6′-*N*-methyl-

TABLE IV. COMPARATIVE *Rf* VALUES OF VF3-1 AND OTHER RELATED ANTIBIOTICS ON PAPER CHROMATOGRAPHY

Solvent system: a lower phase of $CHCl_3$–CH_3OH–17% NH_4OH (2 : 1 : 1).

Antibiotics	*Rf* Values
Antibiotic VF3-1	0.55
Gentamicin C_1	0.59
Gentamicin C_2	0.38
Gentamicin C_{1a}	0.18
Sagamicin	0.49
Sisomicin	0.18
Verdamicin	0.38
Antibiotic G-52	0.49

TABLE V. COMPARATIVE *Rf* VALUES OF VF3-1 AND OTHER RELATED ANTIBIOTICS ON SILICA GEL THIN-LAYER CHROMATOGRAPHY

Solvent system: a lower phase of $CHCl_3$–CH_3OH–conc. NH_4OH (1 : 1 : 1).

Antibiotics	*Rf* C_1 Values[a]
Antibiotic VF3-1	0.90
Gentamicin C_1	1.00
Gentamicin C_2	0.79
Gentamicin C_{1a}	0.63
Sagamicin	0.86
Sisomicin	0.67
Verdamicin	0.86
Antibiotic G-52	0.79

[a] Distance of component from origin/Distance of gentamicin C_1 from origin.

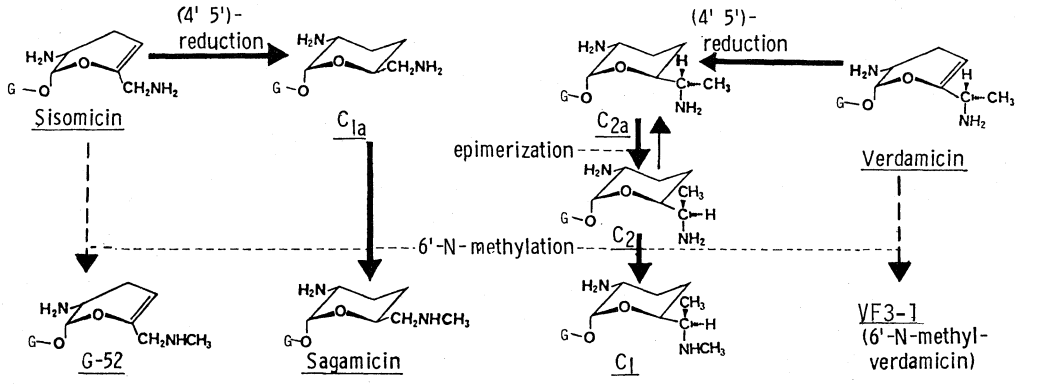


FIG. 7. Biotransformation Sequence of Sisomicin and Verdamicin by *M. sagamiensis* KY 11525 and *M. zionensis* NRRL 5466.

→, *M. sagamiensis* KY 11525; --→, *M. zionensis* NRRL 5466; G, garamine.

verdamicin, although further work will be required to confirm the structure.

DISCUSSION

It was demonstrated that *M. sagamiensis* KY11525 transformed sisomicin to sagamicin *via* gentamicin C_{1a}. No G-52 was detected in the transformation products. As reported previously,[1] gentamicin C_{1a} was transformed to sagamicin. Based on these results, the transformation sequence, sisomicin→gentamicin C_{1a}→sagamicin, was proposed; (4′,5′)-reduction of sisomicin may occur first, followed by the 6′-*N*-methylation (Fig. 7). The results of the time course study supported the above sequence. Similarly, we demonstrated that verdamicin was transformed to gentamicin C_{2a}, C_2, and C_1 by KY11525. No VF3-1 was detected. Gentamicin C_{2a} was transformed to C_2 and C_1, whereas gentamicin C_2 was transformed to C_{2a} and C_1.[1] Mutant KY11565,[1] which appeared to blocked in 6′-epimerase, transformed verdamicin to gentamicin C_{2a} but not to C_2 and C_1. Based on these results and the structure of 4 components (verdamicin, gentamicin C_{2a}, C_2 and C_1), the transformation sequence, verdamicin → $C_{2a} \rightleftharpoons C_2 \rightarrow C_1$, was proposed; the (4′,5′)-reduction of verdamicin occurs first, followed by the 6′-C epimerization and then the 6′-*N*-methylation (Fig. 7).

In contrast with *M. sagamiensis*, *M. purpured*[6] and *M. rhodorangea*,[6] *M. inyoensis* and *M. zionensis* were unable to transform sisomicin to sagamicin. The latter were also unable to transform verdamicin to gentamicin C_{2a}, C_2 and C_1. Alternatively, *M. zionensis* was found to transform sisomicin and verdamicin to G-52 and a new antibiotic VF3-1, respectively. From these results, *M. zionensis* and *M. inyoensis* are proposed to lack the (4′,5′)-reduction activity of sisomicin and verdamicin, and, in *M. zionensis*, direct 6′-*N*-methylation of these antibiotics may occur. The lack of (4′,5′)-reduction activity appears to allow these organisms to produce sisomicin and verdamicin in large amounts. A hypothesis that *M. sagamiensis* and other gentamicin-producing *Micromonospora* also possess the potential activities to synthesize sisomicin-group antibiotics but higher reduction activities in these organisms result in abolishing the productivity of these antibiotics might be reasonably proposed. In fact, sisomicin was reported to be isolated from the minor components in a culture broth of gentamicin-producing *Micromonospora* species.[11] The authors have also isolated sisomicin and antibiotics G-52 from the minor components of a culture broth of sagamicin producing *M. sagamiensis* (unpublished data). Based on the hypothesis, an attempt was made

to derive a mutant of *M. sagamiensis* which was deficient in (4′,5′)-reduction activity and produced a large quantities of sisomicin-group antibiotics. The results will be presented in a series of this paper.

The antibiotic VF3-1 was suggested as 6′-*N*-methylverdamicin by the chromatographic behavior and the mass spectrum. *M. zionensis* appears to possess the 6′-*N*-methylation activity of verdamicin as well as that of sisomicin, although the former activity is very weak.

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