COMMON BIOSYNTHETIC FEATURE OF FORTIMICIN-GROUP ANTIBIOTICS

TOHRU DAIRI and MAMORU HASEGAWA

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machida-shi, Tokyo, Japan

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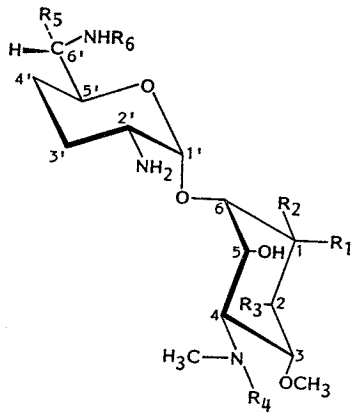
The fortimicin (FTM) group antibiotics are produced by actinomycetes belonging to various genera. FTM's and sannamycins are the products of *Micromonospora olivasterospora* and *Streptomyces sannanensis*, respectively. The possible presence of common features of the biosynthesis of these structurally related antibiotics in the two producers was examined. Enzymatic studies were done by the bioconversion experiments using FTM precursors and the washed cells of *S. sannanensis*. Ion pair/reverse-phase HPLC was employed as the analytical tool. *S. sannanensis* could convert almost all FTM precursors to the expected structures in the down stream of the pathway except one step. Thus the sets of the biosynthetic enzymes of the two bacteria share the common substrate specificity and react alternatively.

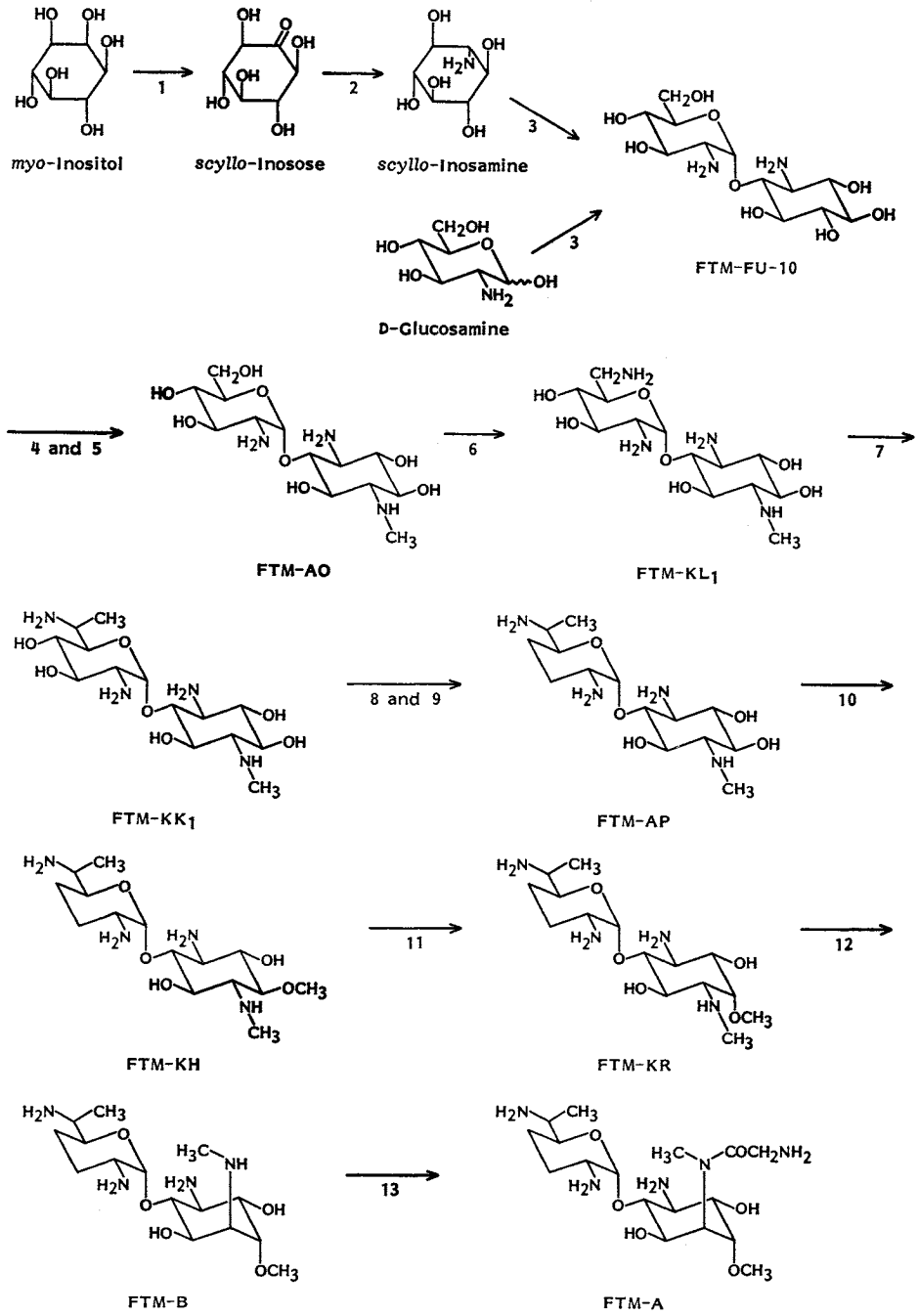
It is known that taxonomically different actinomycetes (on species, or generic level) produce antibiotics of same or very similar structures. It is also the fact that these antibiotics are produced by limited number of strains of each species and/or genera. This 'discontinuous', distribution of antibiotic production resulted in our interest in the problem of evolution and the manner of distribution

Fig. 1. Structures of fortimicin (FTM)-group antibiotics and their producers.



Strain	Product	R_1	R_2	R_3	R_4	R_5	R_6
Micromonospora olivasterospora	FTM-A	NH_2	H	OH	$COCH_2NH_2$	CH_3	H
	FTM-C	NH_2	H	OH	$COCH_2NHCONH_2$	CH_3	H
	FTM-D	NH_2	H	OH	$COCH_2NH_2$	H	H
Micromonospora sp.	SF-2052	NH_2	H	OH	$COCH_2NHCH{=}NH$	CH_3	H
Streptomyces tenjimariensis	Istamycin A	NH_2	H	H	$COCH_2NH_2$	H	CH_3
	Istamycin B	H	NH_2	H	$COCH_2NH_2$	H	CH_3
S. sannanensis	Sannamycin	NH_2	H	H	$COCH_2NH_2$	H	CH_3
Dactylosporangium matsuzakiense	Dactimicin	NH_2	H	OH	$COCH_2NHCH{=}NH$	CH_3	H
Saccharopolyspora hirsuta	Sporaricin A	H	NH_2	H	$COCH_2NH_2$	CH_3	H
	Sporaricin B	H	NH_2	H	H	CH_3	H

Fig. 2. Proposed biosynthetic pathway of FTM-A by *Micromonospora olivasterospora*.



myo-Inositol is oxidized, aminated to form *scyllo*-inosamine, to which D-glucosamine is thought to be linked. Then the product FTM-FU-10 is converted to FTM-AO through 4-amination and 4-*N*-methylation. From FTM-FU-10, -KL_1, -KK_1, -AP, -KH and -KR are successively formed by 6′-amination, 6′-*C*-methylation, 3′,4′-double dehydroxylation, 3-*O*-methylation and 3-epimerization. FTM-B is formed by 4-epimerization, and the glycylation of FTM-B gives FTM-A. See more details in refs 7 and 10.

of secondary metabolism in actinomycetes. What is the difference in genetic background between antibiotic producers and non producers? Do producers have common ancestors, or have they got

the capability to form antibiotics independently during their evolution? Typical examples are so-called fortimicin (FTM)-group antibiotics. In so far as we know, they are produced by actinomycetes of four different genera. FTM's and SF-2052 compounds are produced by *Micromonospora*[1,2], istamycins and sannamycins by *Streptomyces*[3,4], sporaricins by *Saccharopolyspora*[5], and dactimicins by *Dactylosporangium*[6]. In spite of the taxonomically great difference in the producers, these antibiotics possess same or very related structures as shown in Fig. 1. Thus antibiotics of this group are good materials to study the general characteristic nature of antibiotic production described above. The complicated structures and the proposed multi-step pathways of FTM biosynthesis (Fig. 2)[7] seem to suggest the presence of evolutional ancestor. In such a case the biosynthetic pathways, substrate specificities of enzymes involved, and the genetic system of their production are expected to have great community, admitting the presence of some minor modification.

We focused our attention on the biosynthesis of FTM and sannamycin produced by *Micromonospora olivasterospora* and *Streptomyces sannanensis*, respectively. As the first step in the studies of this line, the test was done to see whether the enzymes participating in sannamycin biosynthesis could accept precursors of FTM's as their substrates. As the *in vitro* system for the biosynthetic studies for these antibiotics had not been established, the bioconversion experiments using washed mycelium coupled with analysis by HPLC were employed in this study. Here, we describe the newly developed HPLC method for this assay and the results of bioconversion experiments to show the common nature of these enzyme system of the two bacteria.

Materials and Methods

Strains

S. sannanensis IFO 14239 and a FTM non-producing mutant of *M. olivasterospora* ATCC 21819[1] were used.

Preparation of Washed Cells

Mycelia which had been stocked in 20% glycerol at −80°C were inoculated in 4 ml of seed media, and were grown at 30°C shaking for 3 days. 3 ml of the seed cultures were transferred into 80 ml of production media in 300-ml Erlenmeyer flasks and grown at 30°C for 1 to 3 days on a rotary shaker. The mycelia were collected by centrifugation at 4°C, 6,000×*g* for 10 minutes and washed twice with 0.05 M potassium phosphate buffer pH 7.1. The washed mycelia were resuspended in 8 ml of the same buffer and immediately used for the bioconversion experiments. Seed medium used for *M. olivasterospora* contained (%) Stabilose K (Matsutani Kagaku Kogyo Co., Ltd., Hyogo, Japan) 2.0, glucose 0.5, yeast extract (Nihon Seiyaku Co., Ltd., Tokyo) 0.5, peptone (Kyokuto Seiyaku Co., Ltd., Tokyo) 0.3, meat extract (Kyokuto Seiyaku Co., Ltd.) 0.3, KH_2PO_4 0.02 and $MgSO_4 \cdot 7H_2O$ 0.06 (pH 7.6). Production medium was composed of (%) peptone 0.7, soybean meal 1.5, yeast extract 1.0, glucose 0.5, maltose 4.5, pantothenic acid 0.001, KH_2PO_4 0.1, K_2HPO_4 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, NaCl 0.2 and $FeSO_4 \cdot 7H_2O$ (pH 7.3). Seed medium of *S. sannanensis* had the following composition (%); yeast extract (Difco) 1.2, peptone (Difco) 2.0, malt extract (Difco) 1.2, glucose 1.0 and $CaCO_3$ 0.1 (pH 7.2). Its production medium included (%) Stabilose K 4.0, peptone (Difco) 0.2, corn steep liquor (Nihon Syokuhin Kako Co., Ltd., Tokyo) 0.5, yeast extract (Difco) 0.2, NaCl 0.3, $CaCO_3$ 0.1 and $MgSO_4 \cdot 7H_2O$ 0.05 (pH 7.0). Media were autoclaved at 120°C for 20 minutes.

Bioconversion Experiments

Reaction mixtures contained 0.2 ml each of glycine (9 mg/ml), 2% glucose, and FTM precursors (5 mg/ml) plus 1.4 ml of washed mycelium. The mixtures in test tubes were incubated at 30°C for 20 hours on a reciprocal shaker. To stop the reaction, 0.3 ml of 2 N H_2SO_4 was added, and incubated further for 1 hour. The 10-μl of the supernatant obtained by the centrifugation (at 4°C, 6,000×*g* for

10 minutes) was applied to HPLC.

HPLC Analysis

Analytical HPLC was performed using a Hitachi 655A-12 pump connected with a Hitachi F1100 fluorometer. The post column derivatization of the bioconversion products with *o*-phthalaldehyde (OPA) developed for the analysis of gentamicins by ANHALT[8] was applied here with some modifications. Fluorescence excitation at 365 nm and emission at 490 nm were employed. A column (4.6×250 mm) coupled with precolumn (4.6×10 mm) both packed with Nucleosil 5C 18 (Gasukuro Kogyo Co., Ltd., Tokyo) was used as the stationary phase. Detector signals were processed and recorded by Hitachi D2000 integrator. The mobile phase contained various concentration of Na_2SO_4, methanol, ion pair reagents *i.e.* several kinds of sodium alkyl sulfonate, and 0.1% acetic acid in deionized water. To get the optimum conditions of each FTM precursor, the concentration of Na_2SO_4, methanol and the length of the alkyl chains of the ion pair reagents were varied (Table 1). The flow rate of the mobile phase was 1.5 ml/minute and that of OPA was 0.8 ml/minute. Chromatography and OPA reaction were performed at 56°C.

Table 1. Suitable compositions of mobile phase employed for HPLC analysis of each FTM precursor.

Products to be assayed	Ion pair reagents[a]	Concentration of methanol (%)	Concentration of sodium sulfate (mol)
FTM-FU-10	C-8	5	0.1
FTM-AO	C-7	4	0.05
FTM-KL_1	C-7	5	0.05
FTM-KK_1	C-7	7	0.05
FTM-AP	C-7	8	0.05
FTM-KH	C-7	10	0.05
FTM-KR	C-12	40	0.2
FTM-B, -A	C-7	7	0.2

[a] Carbon numbers of alkyl chains of the reagents are indicated.

Structure Determination of the Bioconversion Products Formed by *S. sannanensis*

FTM-A: The 2-ml of FTM-B (5 mg/ml) was incubated at 30°C for 20 hours in the presence of 2 ml of glycine (9 mg/ml), 2 ml of 2% glucose, and 14 ml of the mycelium suspension of *S. sannanensis*. The supernatant of the reaction mixture obtained as above was adjusted to pH 7.0 with 1 N NaOH and applied on the column (1.0×30 cm) packed with Amberlite CG-50 (NH_4^+ type). After washing with 300 ml of 0.01 M potassium phosphate buffer pH 7.0, FTM's were eluted stepwisely with 50 ml each of 0.005 M, 0.01 M and 0.015 M NH_4OH. Eluants were monitored by TLC. The 10-μl of eluants were spotted on Merck No. 6721 precoated plates and developed in $CHCl_3$ - CH_3OH - NH_4OH (1 : 1 : 1). Spots were visualized by 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD) method[9]. The fractions containing material whose Rf value was 0.6 were collected, and evaporated at 40°C *in vacuo*. The product was dissolved in D_2O and subjected to ^1H NMR analysis.

N-Formimidoyl-FTM-A: The incubation was done as described above except FTM-A (5 mg/ml) was used as the substrate. The neutralized supernatant was applied on the column (1.0×30 cm) packed with Amberlite CG-50 (Mg^{2+}, Na^+ type). After washing with 300 ml of deionized water, FTM's were eluted with 100 ml of H_2O adjusted to pH 4.0 with H_2SO_4. Eluants were applied on the column (1.0×30 cm) packed with Silica gel type C-200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). FTM's were eluted with water adjusted to pH 4.0 with H_2SO_4 into 20 ml fractions. The 10-μl of each fractions was analyzed using HPLC. The fractions containing the product which eluted after FTM-A were collected, and concentrated by evaporation at 60°C *in vacuo*. The product was freeze-dried, dissolved in D_2O and subjected to ^1H NMR analysis.

Results

HPLC Analysis of FTM's

The principle of the separation of HPLC technique originally developed for the assay of gentamicins was reverse-phase, ion pair chromatography[8]. The separation was probably due to the difference in the interaction with hydrophobic stationary phase of the alkyl chains of ion pair reagents which formed complex with positively charged aminoglycosides. The method was employed for the

detection and quantitative analysis of FTM's in this study. By varying the concentration of sodium sulfate, methanol and the length of the alkyl chains of ion pair reagents, we could establish HPLC conditions for all of the FTM biosynthetic precursors who had more than one amino group, namely intermediates after FTM-FU-10† in the pathway (Fig. 2). *myo*-Inositol and *scyllo*-inosose were not retained on the stationary phase. *scyllo*-Inosamine and D-glucosamine were poorly retained but not separated from each other. The resolution of the epimers FTM-KH and -KR were attained only under the strictly critical conditions. Optimum conditions for each intermediate were described in Table 1.

Common Steps of Biosynthesis of Antibiotics between *M. olivasterospora* and *S. sannanensis*

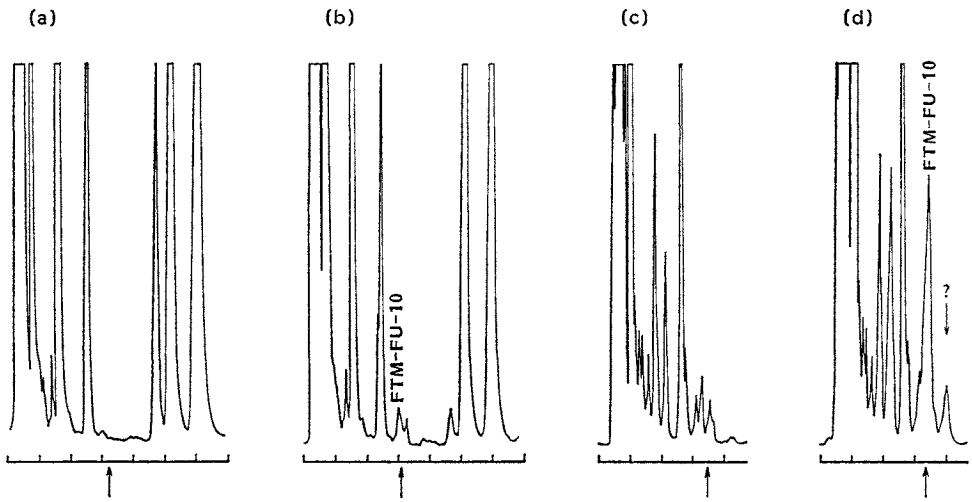
To study the common feature of the antibiotic biosynthesis, we compared the substrate specificity of the enzyme system of the two bacteria, testing if *S. sannanensis* could convert FTM precursors to the successive intermediates through the biosynthetic pathway proposed for *M. olivasterospora* (Fig. 2). The following bioconversion experiments were done using washed mycelia as the catalysts and HPLC technique developed as described above.

Bioconversion of *scyllo*-Inosamine and D-Glucosamine

At this third step of the FTM biosynthesis, D-glucosamine is thought to be introduced to the pathway. Thus this is the introductive step from primary metabolism to the secondary metabolism with respect to D-glucosamine utilization of the cells.

Using *scyllo*-inosamine and D-glucosamine as the substrate precursors, we could detect FTM-FU-10 as the main product of *S. sannanensis* alike the case for *M. olivasterospora* (Fig. 3). The highest

Fig. 3. HPLC analysis of bioconversion of *scyllo*-inosamine and D-glucosamine using washed cells of *Micromonospora olivasterospora* and *Streptomyces sannanensis*.



M. olivasterospora (a and b) and *S. sannanensis* (c and d) were incubated with (b and d) and without the two substrates (a and c).

Retention times were indicated at 5 minutes intervals.

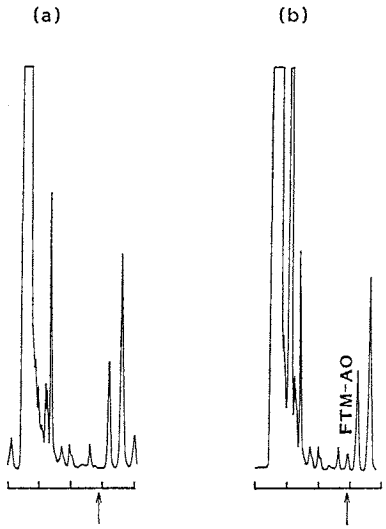
† In this paper intermediates of FTM-A biosynthesis are named in abbreviations like FTM-FU-10, -KL_1, -KK_1, -AP, -KH, -KR, -B and -A. Structures of these compounds are shown in Fig. 2, and in refs 7 and 10.

Table 2. Quantitative analysis of bioconversion experiments from *scyllo*-inosamine and D-glucosamine to FTM-FU-10.

Microorganism	Activity[a]		
	24 hours[b]	48 hours[b]	72 hours[b]
Streptomyces sannanensis	53.1	12.4	0.1
Micromonospora olivasterospora	4.5	2.3	0.1

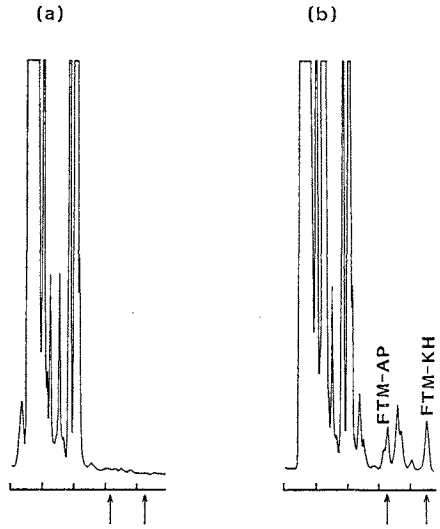
[a] Activity is expressed as FTM-FU-10 formed per dry cell weight (μg/ml, mg).
[b] Cultivation time (hours) of cells used for bioconversion.

Fig. 4. HPLC analysis of bioconversion FTM-FU-10 to FTM-AO using washed cells of *Streptomyces sannanensis*.



S. sannanensis was incubated in the absence (a) and presence (b) of FTM-FU-10.
Retention times were indicated at 5 minutes intervals.

Fig. 5. HPLC analysis of bioconversion of FTM-AO using washed cells of *Streptomyces sannanensis*.



S. sannanensis was incubated in the absence (a) and presence (b) of FTM-AO.
Retention times were indicated at 5 minutes intervals.

activity was found in the mycelia of early stage (24 hours cultivation). This was also the same situation as *M. olivasterospora*. Interestingly *S. sannanensis* possessed much higher enzyme activity than *M. olivasterospora* (Table 2). Beside FTM-FU-10 *S. sannanensis* produced unknown material which appeared at retention time 20.9 minutes.

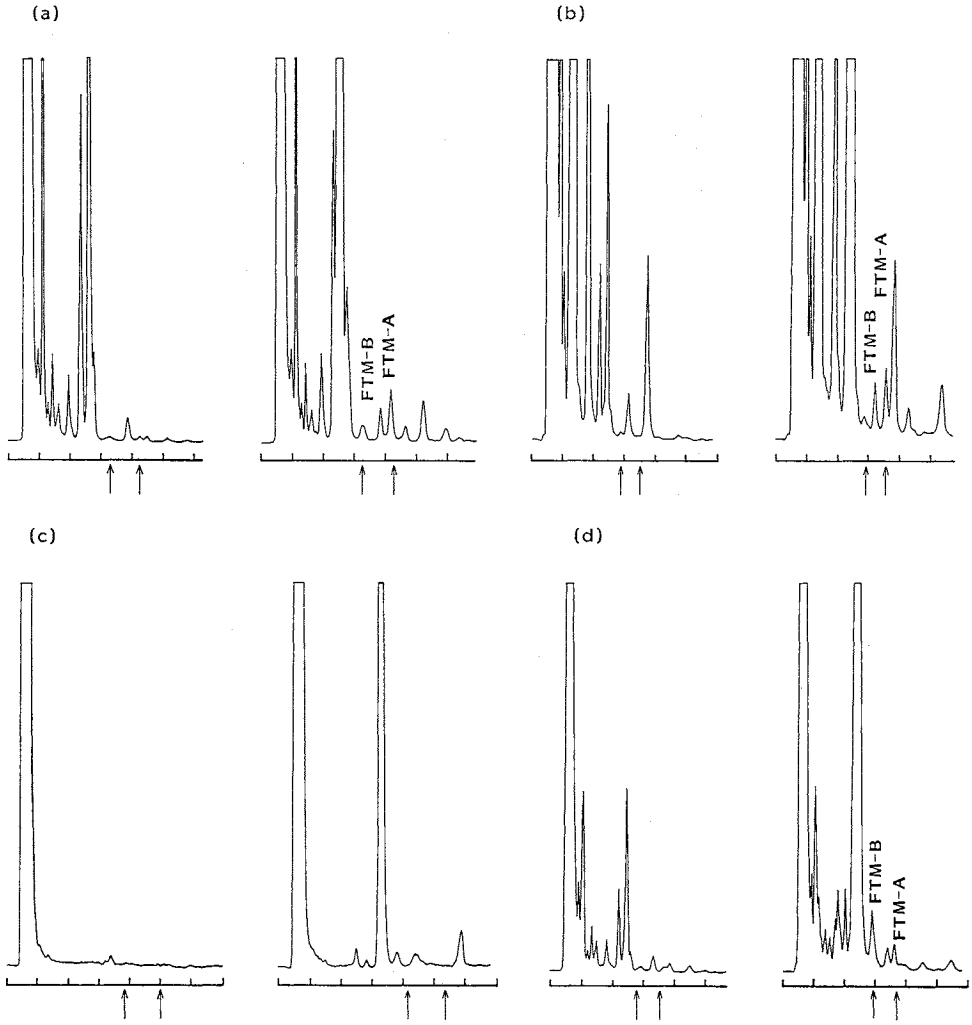
Bioconversion of FTM-FU-10 to FTM-AO (Steps 4 and 5)

These steps involve amination at 4-position of the fortamine moiety and successive *N*-methylation to form FTM-AO. Alike *M. olivasterospora* (not shown), *S. sannanensis* could convert FTM-FU-10 to FTM-AO as shown in Fig. 4. Thus *S. sannanensis* possessed both of the enzymatic activities common to *M. olivasterospora*.

Bioconversion of FTM-AO to FTM-KH (Steps 6~10)

These biosynthetic steps include amination, *C*-methylation, double dehydroxylation at purpurosamine moiety and *O*-methylation of fortamine moiety (Fig. 2). The available substrate to study these steps was only FTM-AO. FTM-KL_1, -KK_1 and -AP in our hands were used as the reference com-

Fig. 6. HPLC analysis of bioconversion from FTM-KH to FTM-KR, and from FTM-KR to FTM-B and -A.



Micromonospora olivasterospora (a and b) and *Streptomyces sannanensis* (c and d) were used. In the absence (left) and presence (right) of substrate FTM-KH (a and c) and FTM-KR (b and d) were shown. Retention times were indicated at 5 minutes intervals.

pounds to monitor HPLC, because of their limited amounts of stocks. Thus we investigated whether we could detect FTM-KL_1, -KK_1, -AP and -KH when FTM-AO was used as the starting material.

Small quantities of FTM-KL_1 and -KK_1 were detected (data not shown). Moreover, relatively large amounts of FTM-AP and -KH were formed in spite of rather long distance from the substrate FTM-AO in the biosynthetic pathway (Fig. 5). When *M. olivasterospora* was used as the catalyst, these intermediates could be detected only when a blocked mutant who did not transform FTM-KH to FTM-KR was used. TLC analysis also support the formation of FTM-KH by *S. sannanensis* (data not shown). In the chromatography, several unknown metabolites could be detected, which were not seen when *M. olivasterospora* was the catalyst. The formation of FTM-KH from FTM-AO by *S. sannanensis* undoubtedly indicates the presence of enzymes which catalyze all the steps (steps

Table 3. Stimulation of formation of FTM-A and *N*-formimidoyl-FTM-A by addition of glycine.

Microorganism	Product	Glycine	Activity[a]
Streptomyces sannanensis	FTM-A	+	69.3
		−	1.7
	N-Formimidoyl-FTM-A	+	54.0
		−	0.3
Micromonospora olivasterospora	FTM-A	+	132.8
		−	3.0

[a] Activity is expressed as FTM-A and *N*-formimidoyl-FTM-A formed per cell weight (μg/ml, mg).

6～10, Fig. 2) of FTM biosynthesis (for precise discussion, see Discussion).

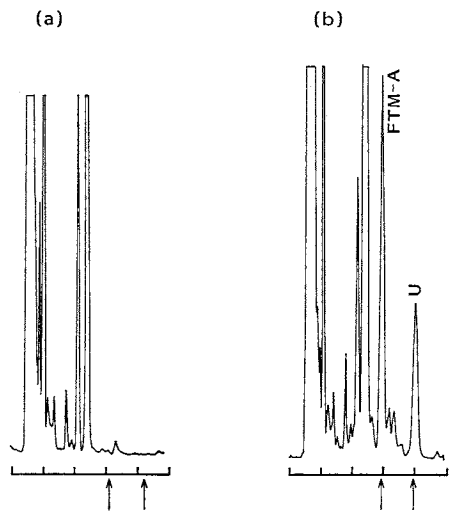
Bioconversion of FTM-KH and -KR

Small quantity of contamination of FTM-KR in the substrate FTM-KH made it difficult to see the bioconversion from FTM-KH. No increase of peak of FTM-KR was detected nor those of FTM-B and -A by *S. sannanensis* as the catalyst. On the other hand, *M. olivasterospora* could form FTM-B and -A from FTM-KH (Figs. 6a and 6c). When FTM-KR was used as the starting material, both *S. sannanensis* and *M. olivasterospora* could form FTM-B and -A (Figs. 6b and 6d). Thus it is strongly suggested that *S. sannanensis* cannot (efficiently) convert FTM-KH to FTM-KR.

Bioconversion of FTM-B

Substrate FTM-B was sufficiently converted to FTM-A by the two bacteria. In the both cases the reaction was greatly enhanced by the addition of glycine (Table 3). Thus FTM-A is thought to be simply synthesized by the combination of FTM-B and glycine.

Fig. 7. Bioconversion of FTM-B by *Streptomyces sannanensis*.



S. sannanensis was incubated in the absence (a) and presence (b) of FTM-B. The unknown peak (U) was identified as *N*-formimidoyl-FTM-A (see text).

Retention times were indicated at 5 minutes intervals.

In this experiment an unknown large peak which eluted after FTM-A was detected when *S. sannanensis* was used as the catalyst (Fig. 7). This unknown product was also formed when FTM-A was used as the substrate (data not shown). The formation of this compound was greatly stimulated by the addition of glycine (Table 3). The compound was isolated and identified as *N*-formimidoyl-FTM-A by ^{1}H NMR which had been reported as the products of *Dactylosporangium matsuzakiense* and *Micromonospora* sp. SF-2098[2,6].

Discussion

In this report the analytical method using HPLC for FTM precursors was accomplished and used for the bioconversion studies. The method had been originally developed for gentamicin analysis, and was modified here for the new aminoglycoside FTM's. With this system the precursor compounds harboring more than one amino groups could be analyzed. *scyllo*-Inosamine and D-glucosamine

who had only one amino group were not efficiently retained on the alkylated stationary phase even if we used longer alkyl chain ion pair reagents (C-6~C-20) in mobile phase of low salt, low concentration of methanol. This situation made it unable to do comparative studies on steps 1 and 2. Analytical conditions for the rest of all biosynthetic steps were established and were applied to research for common biosynthetic steps in *M. olivasterospora* and *S. sannanensis*. Although analytical technique mainly used in this studies was HPLC described above, the proposed structures of the bioconversion products were highly reliable because structurally defined compounds were used as the substrates, and some of products (FTM-FU-10, -KH, -B and -A) were TLC identified. Moreover FTM-A formed from FTM-KR and -B by *S. sannanensis* was undoubtedly identified by ^{1}H NMR spectroscopy.

S. sannanensis could convert most of FTM precursors to various intermediates down stream of the proposed FTM biosynthetic pathway of *M. olivasterospora*. Intermediates *scyllo*-inosamine, D-glucosamine, FTM-FU-10, -AO, -KR and -B were found to be susceptible to the enzymes of *S. sannanensis*. *scyllo*-Inosamine and D-glucosamine were combined to form FTM-FU-10, and then the product could be changed to FTM-AO by *S. sannanensis*. So the enzymes which can catalyze steps 3~5 (Fig. 2) were present in the bacteria. Substrate FTM-AO was also converted to FTM-KL_1, -KK_1, -AP and -KH which were the reaction products of steps 6 to 10, respectively. Steps 8 and 9 include double dehydroxylation reactions at 3′ and 4′ position of purpurosamine moiety. Each step is thought to consist of two successive reactions, dehydration to form carbon-carbon double bond, and then the reduction to form back single bond. Thus at least seven independent reactions which must be present between FTM-AO and -KH in *M. olivasterospora* were proved to be present also in *S. sannanensis*.

Though there was no direct evidence, *S. sannanensis* seemed to lack the ability to transform FTM-KH to FTM-KR (step 11), because no FTM-B nor -A were formed from FTM-KH, but were formed from FTM-KR (Fig. 6). FTM-KH possesses hydroxy group at position 2 of the fortamine moiety whereas correspondent precursor of sannamycin has hydrogen atom at the same position (Fig. 1). The epimerase of *S. sannanensis* which acts on position 3 is thought to be adapted to this 2-dehydroxy type compound. Thus the neighboring bulky hydroxy group of FTM-KH used as the substrate in this experiment might interfere with the active site of the epimerase to attack the methoxy group.

Many unknown peaks were detected when *S. sannanensis* was used as the catalyst. So-called metabolic grid of secondary metabolism and small amounts of their production made it difficult to predict or determine their structures. The unknown product formed when FTM-B and -A were used as the substrates was identified as *N*-formimidoyl-FTM-A. Very small peak of the same retention time on HPLC was also detected when *M. olivasterospora* was used as the catalyst. Furthermore, it is reported that *D. matsuzakiense*, *Micromonospora* sp. SF-2098, *Streptomyces tenjimariensis* and *Saccharopolyspora hirsuta* produced this *N*-formimidoyl type compounds[2,6,11,12]. It is reported that the compound is unstable at pH of alkaline side, and gives FTM-A as the decomposed product[13]. Thus FTM-A isolated from *S. sannanensis* according to the procedure described in Materials and Methods should contain this decomposition product from *N*-formimidoyl-FTM-A. In spite of this complicated situation, the presence of FTM-A itself in the reaction mixtures where FTM-B was used as the substrate is clear (Fig. 7). The formation of free and *N*-formimidoyl-FTM-A was stimulated by the addition of glycine. The biosynthesis of FTM-A from FTM-B and the unique mechanism of the enzymatic formation of *N*-formimidoyl-FTM-A from FTM-A will be reported elsewhere.

In conclusion, *S. sannanensis* possesses enzymes which can catalyze steps 3 to 13 with only one exception (step 11). These enzymes must be involved in the sannamycin production. Thus the biosynthetic pathway for the both antibiotics are common to great extent in *M. olivasterospora* and in *S. sannanensis*. It is highly probable that the enzyme systems for the antibiotic production shares this community in various actinomycetes who produce FTM-group antibiotics. As mentioned before, we supposed the presence of evolutional ancestor for these producers. The enzymatic similarity of the two bacteria revealed in this study seems to support this idea. Comparison of the genetic system of the antibiotic biosynthesis of these bacteria, which will be realized by the gene cloning work might provide us more detailed and deeper insight of this problem. The works of this line are now in prog-

ress.

Recently one member of this antibiotic group, FTM-A has found wide clinical use. It has a broad antibacterial spectrum, and is characterized by remarkably low toxicity compared with other aminoglycoside antibiotics. The observation described here opens new aspect for the production of FTM-A and antibiotics of this group. Most enzymes and hence, genes coding them are exchangeable between these bacteria. So protoplast fusion or recent gene manipulation technique will show their full ability if they are developed on the base of the findings in this study.

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