A NEW ANTIBIOTIC, FUMARAMIDMYCIN*

I. PRODUCTION, BIOLOGICAL PROPERTIES AND CHARACTERIZATION OF PRODUCER STRAIN

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A new antibiotic, fumaramidmycin, has been isolated from a streptomycete NR-7GGl which was characterized and named *Streptomyces kurssanovii*. The strain produced the antibiotic only when grown on agar plates but not in the submerged culture broth, where the contact with the vegetative mycelia appears to cause the inactivation of the antibiotic. The antibiotic shows an antimicrobial activity against both Gram-positive and Gram-negative bacteria.

During studies on screening for antibiotics active against bacteria resistant to various known antibiotics, a new antibiotic with a broad antibacterial spectrum was isolated from the whole agar culture of *Streptomyces* strain NR-7GGl and referred to as fumaramidmycin. This strain was found to produce the antibiotic only on the agar plate.

In this and the subsequent paper[1], the taxonomical characterization of its producer, the production, isolation, biological and chemical properties are described. The chemical structure has been determined to be N-(phenylacetyl)-fumaramide[1]. The name *Streptomyces kurssanovii* NR-7GGl has been assigned to its producer strain which was deposited at the Institute of Industrial Microbiology, Chiba, Japan, as FERM-P No. 2133 in June, 1973.

Materials and Methods

1. Producer and its taxonomy:

Streptomyces sp. strain NR-7GGl was isolated from a soil sample collected in 1972 at Kumamoto City in Japan through cultivation on GGN agar containing glycerol (1 %), glucose (1 %), Polypeptone (1 %), meat extract (1 %), NaCl (0.2 %) and Wako Agar Powder (Lot. No. AD7463) (1.7 %), adjusted to pH 7.0. The cultural characteristics of the microorganism were determined by the methods of WAKSMAN[2] and SHIRLING and GOTTLIEB[3].

2. Media, culture conditions and procedures for isolation and analysis:

GGN medium mentioned above and several other media (some examples are listed in Table 3, see ref. 4 and 5 for detail) were used. A submerged culture was performed with 125 ml of media in a 500-ml Erlenmeyer flask at 27°C for 4~9 days with or without shaking by an Iwashiya RGR No. 2 rotatory shaker (180 rpm). An anaerobic culture was performed in a four-necked flask where the spatial air was substituted by nitrogen gas. The GGN-agar culture for the production of fumaramidmycin was incubated at 27°C for 4~6 days in sterilized, box-type, plates made of stainless steel. The isolation of fumaramidmycin was performed by the

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method described in the subsequent paper[1].

For studies on production mechanism of fumaramidmycin, the mycelium was harvested by centrifugation at 10,000×*g* in the cold in case of the submerged culture (designated as Broth-M). In the case of agar culture, vegetative mycelia were harvested by scraping the plate with a spatula, followed by the centrifugation (designated as Agar-M). Both were washed once with saline and centrifuged (washed mycelia). After incubation of the washed mycelia obtained as above with or without various presumed precursors, the mixtures were extracted with butyl-acetate, concentrated *in vacuo* and separated on TLC by the method described later[1]. The amount of fumaramidmycin purified was determined either by A_{225} absorbancy (ε=23,400,[1]) or by the antibacterial assay on *Escherichia coli* K-12 where the amount was calculated from the inhibitory zone by the use of a calibration curve (150 μg/ml: 15 mm dia., 310 μg/ml: 20 mm dia., linearity range: 40~800 μg/ml). Radioactivity in the spot corresponding to fumaramid-mycin was measured by counting the scraped silica powder placed directly in a scintillation vial or by the use of Packard Chromatoscanner Model 7201. Total protein was determined according to LOWRY[6].

3. Assay and testing procedures:

Toxicity was measured by the intraperitoneal and oral LD_{50} determination in mice. The *in vivo* antimicrobial activity was measured with systemically infected mice or furunculosed trout and expressed as the increase of survival. In the latter case, the antibiotic was given as the admixed feed for 12 days after the infection and then changed to normal feed. The survival was scored during 20 days. The antitumor effect was examined by a simplified bio-assay: After mice received 2×10^5 EHRLICH ascites cells, the treatment was done once a day for 10 consecutive days and the effects were evaluated by the comparison of survival.

4. Test microoganisms:

Antibiotic resistant strains isolated from clinical materials were kindly provided by Prof. S. MITSUHASHI, University of Gunma, Japan. These included *Escherichia coli* CF41, *Staphylococcus aureus* MS 3937, 4018 and 9261 as well as a sensitive strain *E. coli* CF17. Some of microorganisms for MIC determination were generously given by the Institute of Microbial Chemistry, Tokyo.

5. Radioisotopes:

Sodium-2-^{14}C-acetate (49.2 mCi/m mole, Dai-ichi), 2, 3-^{14}C-fumaric acid (59 mCi/m mole, Amersham) and ^{14}C-phenylacetic acid (methylene-^{14}C) (2.04 mCi/m mole, Mallinckrodt) were purchased from Dai-ichi Chem. Co. Ltd.

Fig. 1. Aerial mycelia of *Streptomyces kurssanovii* NR-7GG1 cultured on ISP-4 (a) and on ISP-2 (b) media for 13 days (×200) where the clear formation of spiral in (a) is noted; Electron micrograph of spores of the strain (c) cultured on ISP-3 medium (×5,000)

(a) (b) (c)

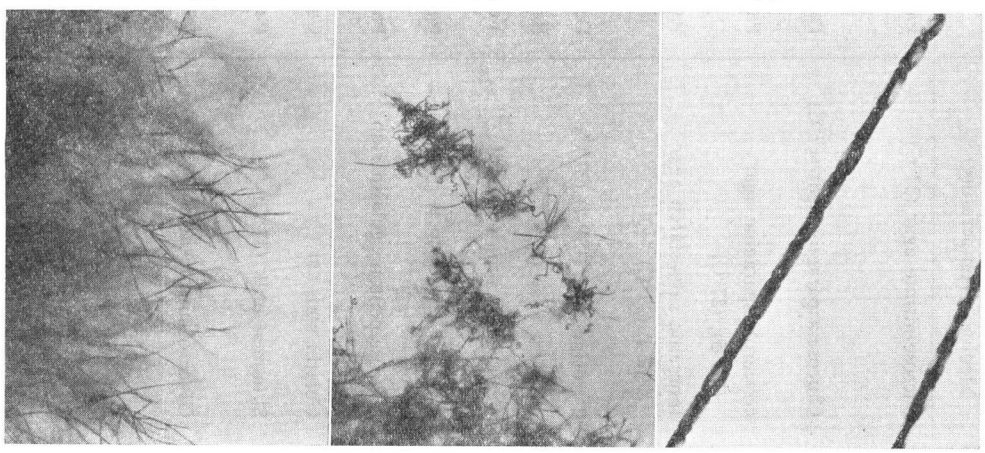


Table 1. Cultural characteristics of *Streptomyces kurssanovii* NR-7GG1

Medium (Temperature)	Vegetative growth	Aerial mycelium	Soluble pigment	Observations
Sucrose-nitrate agar (27°C)	Pale yellowish brown~yellowish brown	Brownish white~light brownish gray [3dc, Natural]	Pale brown	
Glucose-asparagine agar (27°C)	Pale yellowish brown~brownish white	Light brownish gray~light gray	Slightly brownish	
Glycerol-asparagine agar (ISP-5), (27°C)	Pale yellowish brown~yellowish brown	Brownish white~light gray	Slightly brownish	
Inorganic salts-starch agar (ISP-4) (27°C)	Pale yellow~pale yellowish brown	Light brownish gray~brownish gray [3fe, Silver Gray]	None	Hydrolysis of starch (moderate~strong)
Tyrosine agar (ISP-7) (27°C)	Dark gray~dark brown (7 ml)	Scant, white	Blackish	
Nutrient agar (27°C)	Brownish gray	None	Brown	
Yeast extract-malt extract agar (ISP-2) (27°C)	Yellowish brown	Light brownish gray [5fe, Ashes]	Brown	
Oatmeal agar (ISP-3) (27°C)	Pale yellowish brown	Brownish white~light gray [5dc, Pussywillow Gray]	None	
Glucose-peptone gelatin stab (27°C)	Pale yellowish brown~yellowish brown	Brownish white	Dark brown	Liquefaction (mild after 10th days)
Gelatin stab (20°C)	Colorless	White~brownish white	Brown	Little or no liquefaction
Skimmed milk (37°C)	Pale yellowish brown~yellowish brown	None	Brown	No coagulation, complete peptonization (at 10th day)
Glycerol-nitrate agar (27°C)	Pale yellowish brown~yellowish brown	Brownish white~light brownish gray [2dc, Natural]	Brown	Nitrate reduction negative (ISP-8)
Starch agar (27°C)	Pale yellow~dull yellow [1 1/2 ea. Lt. yellow]	Thin, brownish white	Slightly brownish	
Calcium malate agar (27°C)	Colorless	Scant, white	None	Solubilization (slight)
Cellulose (27°C)	No growth			

Results and Discussion

1. Taxonomical Characterization of the Strain NR-7GG1

On most solid media, the strain NR-7GG1 developed abundant, relatively long, aerial mycelia originating from well-branched, vegetative mycelium. The spiral formation was observed clearly on an inorganic salts-starch agar (ISP-medium 4)[2] (Fig. 1b), but not recognized on most of other media used, even when the aerial mycelium was abundantly developed (Fig. 1a). Whorl formation was not observed with this strain. When matured, spores were arranged in chains of more than 10. Electron micrographs of spores exhibited a flat and smooth surface (Fig. 1c), the dimensions of which were 0.4~0.6 μ by 1.2~1.5 μ.

Growth characteristics of strain NR-7GG1 were observed on cultures incubated at 27°C

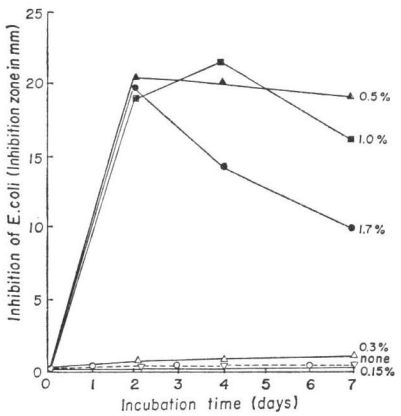
Table 2. Comparison of cultural characteristics among three closely related strains

Characteristics	NR-7GG1	*A. Kurssanovii* ISP 5162	*S. narbonensis* ISP 5016
Color and morphology of aerial mycelium	Brownish white~light brownish gray Spirals Marked spiral formation on ISP medium-4, long aerial mycelium, more than 10 spores in chain	Brownish white~light brownish gray Retinaculiaperti Hooks, loops, short aerial mycelium, 3-10 spores in chain	Brownish white~light brownish gray Rectiflexibiles
Surface of spore	Smooth	Smooth	Smooth
Color of vegetative growth	Yellowish brown	Yellowish brown	Pale yellowish brown
Melanoid formation on			
ISP med. 1	+	−	+
ISP med. 6	+	+	+
ISP med. 7	+	+	−
Milk coagulation	−	−	−
Peptonization	+	+	+
Utilization of carbohydrates			
L-Arabinose	+	+	+
D-Xylose	−	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
Sucrose	+	+	+
Inositol	−	+ (7,8)	−
L-Rhamnose	−	−	±
Raffinose	+	+	+
D-Mannitol	−	−	−
Antimicrobial activity	Fumaramidmycin	An antibiotic active against Gram-positive bacteria and *Saccharomyces* sp.	Narbomycin[11]

for 21 days on various agar media in a cross-hatched pattern. Color names, recorded for mature culture, are listed according to Color Harmony Manual issued from Container Corporation of America, Chicago. The results are summarized in Table 1. Positive growth on glucose-asparagine agar was observed at 20°, 24°, 27°, 30° and 37°C but not at 50°C. Optimum temperature was approximately 30°C. These studies revealed that the strain NR-7GGl, belonging to genus *Streptomyces*, has the characteristics shown in Table 2 (left column). When these descriptions were compared with those of known species of actinomycetes[2,3,7~9], a good agreement was found with the following two strains: *Actinomyces kurssanovii* ISP 5162[7,8] and *Streptomyces narbonensis* ISP 5016[3,9] (Table 2). Distinct differences between NR-7GGl and *S. narbonensis* were clearly evidenced in the morphology of aerial mycelium, melanoid formation on ISP-medium 7 and the utilization of L-rhamnose. On the other hand, the strain NR-7GGl closely resembled *A. kurssanovii* except for the morphology of aerial mycelium. Namely, the former exhibits a marked spiral formation on ISP-4 agar and bore chains of more than 10 spores on the relatively long aerial mycelia. On all ISP media used, on the other hand, aerial mycelium of *A. kurssanovii* was relatively short in length and bore spore chains with 3~10 spores. According to the original description[8] of *A. kurssanovii*, it was described to be of "spiral formation". This could mean that the strain would form a clean spiral under an appropriate condition. On the basis that the slight differences observed suggest the close relation to *A. kurssanovii* and that it is clear the strain belongs to genus *Streptomyces* according to contemporary criteria[10], we have named the strain *S. kurssanovii* NR-7GGl.

Fig. 2. Time course of antibiotic activity produced under various culture conditions

An aliquot (0.3 ml) of 125 ml of shaken culture (shown by open symbols) in the absence or the presence of Wako agar was placed in an assay cup after the centrifugation of the broth at 10,000×*g* for 10 minutes. A plate culture (shown by solid symbols) of varying agar concentration received NR-7GGl as evenly as possible. At intervals, the agar from 2 cultured Petri dishes (10 cm dia., 15 ml GGN agar) was put into a 500-ml shaker flask and shaken overnight at 27°C with 100 ml of methanol in a rotary shaker. The methanol extracts were filtered and concentrated *in vacuo* into 1.5 ml, 0.3 ml of which was placed in the cup. The inhibitory zone was measured after 18 hours incubation at 37°C using *E. coli* K-12 as the test organism. Mycelial growth: ##, 1.7%, plate; #, all others.



2. Production of Fumaramidmycin under Various Culture Conditions:

By the agar-cross streak assay, the culture of *S. kurssanovii* NR-7GGl was found to produce a broad spectrum antibacterial active substance (we designated as fumaramidmycin) on GGN agar. Figure 2 shows the time course of the antibiotic production by the strain cultured in GGN medium under various cultural conditions. It is clear that this strain shows the activity only on the agar plate, after 2 days of incubation at 27°C. The appearance of the antibacterial activity was found indifferent with regard to the grade of mycelial growth (not shown). Attempts to make the strain produce the antibiotic in a submerged culture were unsuccessful. Either

Table 3. Effect of the growth condition on the antibacterial activity of *S. kurssanovii* NR-7GG1 strain

Medium	Culture form	Days	pH	Pigmentation	Inhibitory zone (mm dia.) *vs.*			
					E. coli	*Staphylococcus aureus* 209P	*Pseudomonas aeruginosa*	*Bacillus subtilis*
Fumaramidmycin	420 μg/ml purified (from GGN-A 1.7, 5 days)				22	18	0*	20
GGN-A 1.7[a]	plate	5	<5.8	++	19	17	t	17
GGN-A 0.3	shaking	4	<5.8	++	0	0	t	12
GGN-ag[b] 0.3	shaking	4	<5.8	+	0	0	t	10
GGN-gel[c] 1.0	shaking	4	<5.8	±	0*	0	t	12
GGN	shaking	2	6.8	++	0*	0*	11	11
GGN	shaking	5	8.6	+++	0*	0	t	11
GGN	standing	7	5.8	++	0*	0*	14	15
GGN-N_2	standing	7	6.2	+	0*	0*	12	16
B[d]	shaking	4	>8	+++	0	0	0	5
	standing	7	7.2	++	0*	0*	14	17
D[e]	standing	7	5.8	++	0*	0*	15	18
W[f]	standing	7	6.4	++	0*	0*	13	13

a) A 1.7; Agar powder (Wako) at the concentration of 1.7 %
b) ag; agarose A-37 (Nakarai)
c) gel; Bacto-gelatin
d) B; a glycerol-yeast extract-medium[4]
e) D; glucosestarch-soya bean medium[5]
f) W; WAKSMAN's medium containing glucose (1 %), meat extract (1 %) and Polypeptone (0.02 %) pH 7.0

Method: Culture (125 ml) was applied for the cup assay after the filtration of centrifugation at 10,000×*g* for 10 minutes at 0°C except GGN-A 1.7 plate where 3 liters of the agar culture was extracted with 3 liters MeOH by overnight stirring at room temperature.

*; incomplete inhibition:
t; trace means the complete inhibition within the cup area
+~+++; grade of pigmentation which is an indicator of the extent of mycelial growth.
N_2; Air was substituted by nitrogen gas.

shaking or standing culture in more than 15 different media, as well as an anaerobic culture in GGN without shaking, yielded no activity against *E. coli*. A part of those results is shown in Table 3. When the mycelia obtained from 6-day shaking or standing culture in GGN were extracted with methanol followed by TLC, no spot identical with fumaramidmycin was detected on TLC[2], indicating that agar plate-dependent production was not due to the reduced aeration. Instead, in shaking culture, a slight antibiotic activity was seen to appear against *Bacillus subtilis* and *Ps. aeruginosa*, indicating, together with difference in the behavior to the solvent extraction (not shown), that the strain produces at least one more antibiotic of different nature from that of fumaramidmycin when grown in the submerged culture. Figure 2 and Table 3 also suggest that not the presence of the agar in the culture but the solidity of the culture or the separation of the product from the mycelia, may have a greater importance to the production of fumaramidmycin, since in the shaken culture the addition of 0.3 % agar or agarose did not improve the activity of culture filtrate.

Table 4. Fumaramidmycin produced by incubation with washed mycelia

	Addition*	Concentration (mM)	Fumaramidmycin(μg/ml reaction mixture) produced			
			by Agar-M**		by Broth-M**	
			4 hours	19 hours	4 hours	19 hours
1.	None		0	23	t***	31
2.	PN	6.4	19	37	19	42
3.	PA	6.4	9	t	t	26
4.	PN+Fam	6.4+12.8	26	69	25	83
5.		6.4+25.6	103	17	46	31
6.		6.4+51.2	32	17	23	37
7.	PA+Fam	6.4+25.6	t	14	57	21
8.		6.4+51.2	23	9	31	34
9.	PN+Famm	6.4+25.6	52	14	34	22
10.		6.4+51.2	20	20	32	20
11.	PA+Famm	6.4+25.6	3	13	33	26
12.		6.4+51.2	0	6	26	34
13.	PN+FA	6.4+25.6	46	20	20	20
14.		6.4+51.2	11	48	50	60
15.	PA+FA	6.4+25.6	t	13	34	37
16.		6.4+51.2	9	0	49	52

* Incubation mixture (total 3.0 ml) contained per ml: one third parts of nutrient broth, pH 6.0; 66 μl of mycelium obtained from agar culture or submerged culture 6.4 μmole of phenylacetamide (PN) or phenylacetic acid (PA), and 12.8, 25.6 or 51.2 μmole per ml of fumaramic acid (Fam), fumaramide (Famm) and fumaric acid (FA) was added in addition.

** Total protein: Agar-M, 29.4 μg/ml; Broth-M, 31.2 μg/ml reaction mixture. For the definition of Agar-M and Broth-M see Materials and Methods.

*** t means trace, positive but subject to error in determination, estimated <5 μg. Amount of fumaramidmycin was determined spectrophotometrically from A_{225} of the spot on TLC. More than two determinations were done only in experiments 1~4, where the standard deviation as 5~16 %.

3. Mechanism of Fumaramidmycin Production

In an attempt to clarify why fumaramidmycin was not detected in submerged culture, the capacity to produce the antibiotic was compared between the mycelia obtained from GGN agar culture (Agar-M) and that from the submerged culture in GGN medium (Broth-M). When freshly prepared mycelia were shaken in a fresh nutrient broth (Eiken, adjusted to pH 6.0) for 19 hours at 27°C, a minute but significant amount of fumaramidmycin was detected on TLC[1] as shown in Table 4. No significant difference of this capacity was observed between Agar-M and Broth-M. The addition of phenylacetamide or of phenylacetic acid, presumed precursors, was found to enhance the production of the antibiotic. Further addition of other presumed precursors, fumarate derivatives, appeared to increase the production. It is noted that phenylacetamide and phenylacetic acid almost equally stimulated the production by Broth-M, while the production by Agar-M was not so much increased by the addition of phenylacetic acid (Table 4). In some cases larger amounts of fumaramidmycin were detected in 4-hour incubation than in 19-hour incubation, suggesting that degradation of fumaramidmycin occurred during the prolonged incubation. When Agar-M or Broth-M was shaken in nutrient broth with ^{14}C-

Table 5. Incorporation of labeled fumarate and phenylacetic acid into fumaramidmycin during incubation with Agar-M and Broth-M

Precursors	Amount added (μ moles per reaction mixture)	Mycelial volume (μ l)	Incorporation into fumaramidmycin	
			with Agar-M n moles incorporated	with Broth-M n moles incorporated
[2,3-^{14}C] Fumaric acid	0.17	45	10	12
	12.9	22.5	25	110
	12.9	45	29	260
	25.9	22.5	170	160
	25.9	45	140	360
[Methylene-^{14}C] Phenylacetic acid	0.49	45	150	180
	3.74	22.5	160	220
	3.74	45	160	220
	6.99	22.5	270	400
	6.99	45	400	490

* Total 1 μCi of each compound in various specific activities was incubated with Agar-M or Broth-M in nutrient broth, pH 6.0, by shaking at 27°C (total mixture was 3.0 ml). Agar-M and Broth-M contained 1.13 mg and 0.55 mg protein per ml of packed volume, respectively. When 0.17 μmole of fumaric acid was added, 1.3×10^8 dpm corresponds to 10 n mole incorporated, while in case of 0.49 μmole of phenylacetic acid 7.1×10^5 dpm corresponds to 150 n mole.

Table 6. Degradation of fumaramidmycin by washed mycelia

Presence of fumaramidmycin*	Mycelia	Incubation (19 hours) at	Fumaramidmycin detected μg/ml	Amount degraded μg/ml**	%Degradation
+ (control)	None	0°C	260	0	—
+ (control)	None	27°C	180	80	0
−	A	27°C	23	—	—
+	A	27°C	166	117	14.2
−	B	27°C	30	—	—
+	B	27°C	68	222	54.5

* Fumaramidmycin (261 μg/ml) was incubated in nutrient broth, pH 6.0, for 19 hr at 27°C in the presence or absence of 67 μl per ml of Agar-M (A), Broth-M (B) obtained from doubled mycelial packed volume. Total volume of reaction mixture was 3.0 ml. Protein content of A and B was 29.4 and 31.2 μg per ml reaction mixture, respectively.

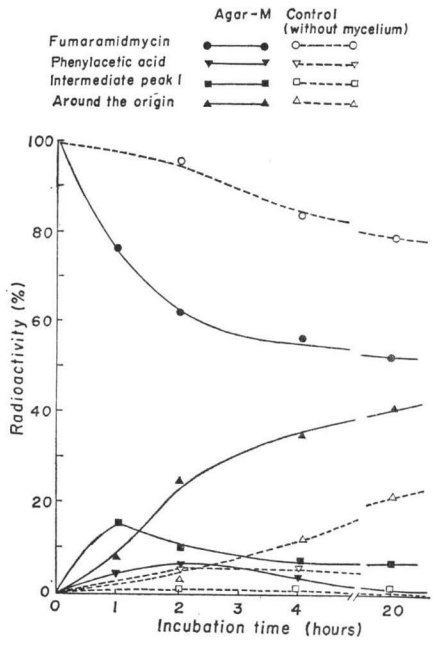
** Amount of fumaramidmycin synthesized by incubation in the absence of fumaramidmycin was subtracted from the amount detected.

*** Percent of real amount degraded (apparent amount degraded minus "control, 27°C" value) to the amount added ("control, 0°C value).

phenylacetic acid or ^{14}C-fumaric acid under the same condition, the radioactivity was found to be incorporated into the isolated fumaramidmycin on TLC as shown in Table 5. Again no remarkable difference of the incorporation efficiency was seen between Agar-M and Broth-M on the basis of packed volume of mycelia, while Broth-M showed a higher incorporation than Agar-M per protein basis. From these results, it was concluded that Broth-M has a similar capacity per mycelial volume basis, or even higher capacity per cellular protein basis, of fumaramidmycin biosynthesis than does Agar-M.

As suggested by the above experiments, the degradative activity of both mycelia (Agar-M and Broth-M) was compared while shaking in nutrient broth. All shaking experiments of fumaramidmycin with mycelia were performed at pH 5.8~6.0 because of its instability at alkaline pH as well as at pH lower than 5[1]. Table 6 shows that Broth-M had about three times stronger activity to degrade it than did Agar-M, when compared after subtraction of newly produced amounts in the presence of mycelium alone. The fact that Agar-M does have degrading activity for the antibiotic was confirmed by incubation with the labeled fumaramidmycin which had been obtained by culturing the strain for 5 days with ^{14}C-acetate (Fig. 3). Figure 3 shows that Agar-M actually degraded about 50 % of fumaramidmycin within 4 hours and the "degradation" apparently ceased thereafter, reaching a plateau. The further addition of a new batch of Agar-M caused further degradation (not shown). A similar pattern of degradation was observed with Broth-M (not shown), suggesting a kind of equilibrium in the concentration of fumaramidmycin and the degradation systems.

Fig. 3. Time course of fumaramidmycin degradation by the incubation with Agar-M
Fumaramidmycin labeled with ^{14}C-acetate was incubated with 15 μl of Agar-M (17 μg protein) in 0.5 ml of NB, pH 6.0 (total 19.800 dpm, *ca.* 400 μg fumaramidmycin). At intervals, a portion (0.1 ml) was taken out and extracted with butylacetate. The concentrated extract was quantitatively spotted on TLC. After developing[1], the radioactivity at the spot of fumaramidmycin, phenylacetic acid, intermediate peak I or around the origin was determined and expressed as the percent of the initial radioactivity. Labeled fumaramidmycin with ^{14}C-acetate was obtained from total 150 ml of GGN agar plates[1], cultured at 27°C for 5 days in the presence of 50 μCi of sodium [2-^{14}C] acetate, by the isolation method previously described.



Evidence obtained in this study clearly shows that mycelium of *S. kurssanovii* NR-7GGl has a similar capacity to synthesize fumaramidmycin regardless of being cultured on GGN agar or in GGN broth. However, the mycelium in the submerged culture has a stronger inactivating activity for the produced antibiotic than that obtained from cultured agar. Therefore it is indicated that, in submerged culture, fumaramidmycin, even if produced, could not be detected by the cup assay because of mycelial inactivation. On the other hand, fumaramidmycin produced on agar would diffuse freely into the agar plate and therefore be able to escape from the mycelial degradation by failing to recontact vegetative mycelia which are immobilized at the inoculated place on the agar plate. Weaker degradation activity of Agar-M may be an additional factor resulting in a higher production on the agar plate than in the submerged culture. The preliminary survey of the components involved in this study suggests a possible pathway of fumaramidmycin biosynthesis *via* phenylacetamide and

Table 7. *In vitro* antimicrobial activity of fumaramidmycin

Test microorganism		M.I.C. (mcg/ml)	Medium
Pseudomonas aeruginosa	IFO 12689	200	1
Pseudomonas aeruginosa	A3	200	1
Pseudomonas fluorescence		>200	2**
Alcaligenes faecalis	IAM 1015	100	2
Escherichia coli	NIHJ	25	2
	K12	37. 5	1
	K12 ML1630 *(CM, KM, NM, SM, TC)	37. 5	1
	W677	100	2
	JR66/W677 *(AB-PC, CEP, GM, KM, NM, SA, SM, TC)	50	2
	CF17	75	1
	CF41 *(AB-PC, CM, KM, SA, SM, TC)	75	1
Citrobacter sp.	KMC-53 *(KM, SA, SM)	100	2
Salmonella typhimurium	1406	37. 5	1
Shigella flexineri	4b JS1181	50	2
Shigella sonnei	JS11746	50	2
Klebsiella pneumoniae	PCI 602	37. 5	1
Enterobacter aerogenes	171-4	100	2
Serratia marcescens	IFO 12648 *(KM, SA, SM)	>200	2
Proteus vulgaris	OX19	25	2
Vibrio anguillarum	NCBM-6 (Eel)	3.12	2**
Aeromonas salmonicida		6.25	2**
Aeromonas punctata	IAM 1646	12. 5	2**
Aeromonas sp.	KT 444	12. 5	2**
Micrococcus flavus	FDA 16	6.25	2
Staphylococcus aureus	FDA 209P	37. 5	1
	Smith	50	2
	MS 3937 *(KM, SA, TC)	12. 5	1
	MS 4018 *(CM, PC, SA, TC)	18.75	1
	MS 9261 *(Mac, PC, SA, TC)	6.25	1
Sarcina lutea	PCI 1001	3.12	2
Bacillus subtilis	PCI 219	37. 5	1
Bacillus anthracis		25	2
Corynebacterium bovis	1810	25	2
Mycobacterium smegmatis	ATCC 607	50	2
Candida albicans	3147	>200	2

Starred* strains are resistant against various known antibiotics in parenthesis: AB-PC, ampicillin; CEP, cephalosporin; CM, chloramphenicol; GM, gentamicin; KM, kanamycin; Mac, macrolides; NM, neomycin; SA, sulfonamides; SM, streptomycin; TC, tetracyclins. By the agar dilution method with 1 (Heart infusion agar) or 2 (nutrient agar), judged after 17 hours incubation at 37°C or at 27°C (**).

fumaramic acid.

4. Biological Properties of Fumaramidmycin

In vitro antimicrobial activities of fumaramidmycin are shown in Table 7. The minimal inhibitory concentration (MIC) was determined with 5% DMSO solution, since fumaramidmycin

is practically insoluble in all solvents but DMSO. As indicated in Table 7, the antibiotic was moderately active against most Gram-negative and Gram-positive bacteria including those resistant to common antibiotics; although the MIC value itself was rather high for practical use except for that against several fish pathogens like aeromonads and *Vibrio anguillarum*. This antibiotic showed no inhibition of fungi or yeast (*Candida albicans*). In 20% DMSO solution, its acute toxicity in mice was found to be 75~100 mg/kg as an LD_{50} does with intraperitoneal and 1.25~2.5 g/kg with oral administration. When examined in systemic infection of mice with *E. coli* 1346, *Ps. aeruginosa* BA and *Streptococcus pyogenes* β-15, the oral administration of fumaramidmycin proved to be inactive *in vivo* at one tenth the LD_{50}. On the other hand, when trout furunculosis caused by a moderate infection with *A. salmonicida* was treated with fumaramidmycin-containing feed, the administration of 900 ppm for 12 days per bath resulted in a small increase of survival (Average fish weight 45 g. Dead/Total: control, 25/50; 100 ppm of fumaramidmycin, 22/50; 900 ppm of fumaramidmycin; 12/50). *In vivo* antitumor test with Ehrlich ascites carcinoma revealed no life-prolongation activity of this antibiotic.

Added in Proof

After we presented the work orally at the 95th Annual Meeting of the Japanese Society for Pharmaceutical Sciences (Apr. 6, 1975 at Nishinomiya, p. III-53 & II-273 of the Abstract issued in Feb. 1975), we were informed that HASEGAWA *et al.* of Takeda Chemical Industries, Ltd. had found the same compound named as C-9154 in the culture broth of a new species of *Streptomyces*, the study of which was submitted to J. Antibiotics in March, 1975.

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