STUDIES ON NEW AMINOGLYCOSIDE ANTIBIOTICS, ISTAMYCINS, FROM AN ACTINOMYCETE ISOLATED FROM A MARINE ENVIRONMENT

III. NUTRITIONAL EFFECTS ON ISTAMYCIN PRODUCTION AND ADDITIONAL CHEMICAL AND BIOLOGICAL PROPERTIES OF ISTAMYCINS

KUNIMOTO HOTTA, MAKOTO YOSHIDA, MASA HAMADA and YOSHIRO OKAMI

Institute of Microbial Chemistry
14–23 Kamiosaki 3-Chome, Shinagawa-ku, Tokyo 141, Japan

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Streptomyces tenjimariensis SS-939 produced istamycins in a medium containing starch as the carbon source and soy bean meal as the nitrogen source. Istamycin production decreased substantially when starch was substituted with mono- or di- saccharides such as glucose, glycerol and maltose. A marked decrease of istamycin production was also observed when a rapidly used nitrogen source such as yeast extract, peptone or casamino acid was employed instead of soy bean meal. Addition of palmitate at a concentration of 0.2% doubled istamycin production. Istamycins A and B were found to be as active as fortimicin A and sporaricin A against Gram-positive and Gram-negative bacteria including aminoglycoside-resistant strains.

As previously reported[1], a mixture of new aminoglycoside antibiotics, istamycins, was found to be produced by a new *Streptomyces* species, *Streptomyces tenjimariensis* SS-939, in the course of screening for new antibiotics. Istamycins were described in terms of their isolation and structures[1], and the istamycin-producing strain and its plasmids were characterized[2]. It was also found that chemical factors such as palmitate and glucose had a significant influence on istamycin production by strain SS-939 and its acriflavine-treated mutants[3]. In this paper the influence of nitrogen sources and carbon sources, including palmitate, on istamycin production in strain SS-939 will be reported. Some additional chemical and biological properties of istamycins A and B will be also described.

Materials and Methods

Fermentation

Streptomyces tenjimariensis SS-939, grown on an inorganic salts-starch agar[4] slant at 27°C for 10 days, was transferred into 20 ml of fermentation medium in 100 ml Erlenmeyer flasks and cultured at 27°C for 3~5 days on a rotary shaker at 180 r.p.m. The basic medium consisted of the following ingredients: potato starch 2.0%, soy bean meal (Prorich S, Ajinomoto Co.) 2.0%, glucose 0.2%, sodium palmitate 0.2%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1% and K_2HPO_4 0.1%. Total istamycins were estimated by diluting and mixing the filtrate of the cultured broth with M/15 phosphate buffer (pH 6.8) and assaying as istamycin A by the cylindrical cup method with *Bacillus subtilis* PCI 219 as the test organism.

Minimum inhibitory concentrations of istamycins A and B

The minimum inhibitory concentrations of istamycins A and B affecting Gram-positive and Gram-negative bacteria, including those resistant to aminoglycoside antibiotics, were determined in nutrient agar by the two-fold serial dilution method and compared to those of fortimicin A and sporaricin A.

Results

Effect of Medium Composition on Istamycin Production

Table 1 shows the effect of various carbon sources on istamycin production. Istamycin production was greatly influenced by the carbon source, although the organism grew well in all the media. Among carbon sources tested, polysaccharides such as starch and dextrin gave good istamycin production. Disaccharide and monosaccharide carbon sources however, exerted an inhibitory effect, suggesting possible carbon catabolite regulation. The fermentation proceeded slowly with moderate istamycin production when soy bean oil or palm oil was added. In these media the pH also went up slowly compared with the other media (data not shown).

Istamycin production was also markedly influenced by nitrogen source, as shown in Table 2. Relatively high yields of istamycin were obtained only when soy bean meal or corn gluten meal were added at a concentration of 2%. Addition of yeast extract, peptone, casamino acid, NZ-amine and meat extract inhibited istamycin production, although growth was not inhibited. This suggests that nitrogen catabolite regulation of istamycin production occurs.

The combined effect of starch and soy bean meal at various concentrations was then examined as shown in Table 3. The higher concentration of soy bean meal gave a more rapid pH rise of the media during incubation. The rise of pH, however, was delayed when the concentration of starch was increased. The highest concentration of istamycin was obtained with a medium containing 2.0% soy bean meal and 2.0% potato starch. Although abundant growth was observed with 3.0% soy bean meal, the culture broth became a highly

Table 1. Effect of carbon sources on istamycin production.

Carbon source	%	Istamycin* (μg/ml)	
		3 days	4 days
Starch Potato	2.0	29.0	33.0
Corn	2.0	29.0	33.0
Soluble	2.0	19.0	40.0
Dextrin	2.0	14.0	28.0
Maltose	2.0	tr	tr
Sucrose	2.0	3.0	tr
Glucose	2.0	tr	0
Inositol	2.0	5.0	tr
Glycerol	2.0	tr	0
Glucosamine	1.0	tr	0
Soy bean oil	1.0	7.0	15.0
Palm oil	1.0	7.0	17.0
No addition		4.0	tr

Basal medium: Soy bean meal (Prorich S) 2.0%, glucose 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.3%, and sodium palmitate 0.2%; pH 7.0.
* Amount of istamycin produced was expressed as istamycin A.

Table 2. Effect of nitrogen sources on istamycin production.

Nitrogen source	%	Istamycin* (μg/ml)	
		3 days	4 days
Soy bean meal (Prorich S)	1.0	15	17
	2.0	25	32
Cotton seed meal (Pharma media)	1.0	tr	2
	2.0	8	12
Corn gluten meal	1.0	5	23
	2.0	12	28
Corn steep liquor	0.5	tr	tr
	1.0	tr	2
Yeast extract	0.5	7	4
	1.0	tr	0
Peptone	0.5	0	0
	1.0	0	0
Casamino acid	0.5	0	0
	1.0	0	0
NZ-Amine	0.5	0	0
	1.0	0	0
Meat extract	0.5	9	6
	1.0	tr	0

Basal medium: Potato starch 2.0%, glucose 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.3% and sodium palmitate 0.2%.
* Istamycin was estimated as istamycin A.

Table 3. Combined effect of starch and soy bean meal on istamycin production.

Concentration (%) of		pH		Potency***	
SBM*	Starch*	3**	4**	3**	4**
1.0	0.5	7.4	7.6	2.5	9.0
〃	1.0	7.4	7.6	2.0	25.0
〃	2.0	7.0	7.6	3.0	19.0
〃	3.0	7.0	7.4	2.0	9.0
2.0	0.5	8.2	8.2	3.0	10.0
〃	1.0	8.0	8.2	9.0	19.0
〃	2.0	7.6	7.8	9.0	67.0
〃	3.0	7.6	7.8	5.0	41.0
3.0	0.5	8.2	8.2	tr	1.5
〃	1.0	8.2	8.2	2.0	3.0
〃	2.0	8.2	8.2	3.0	15.2
〃	3.0	7.6	7.8	tr	6.0

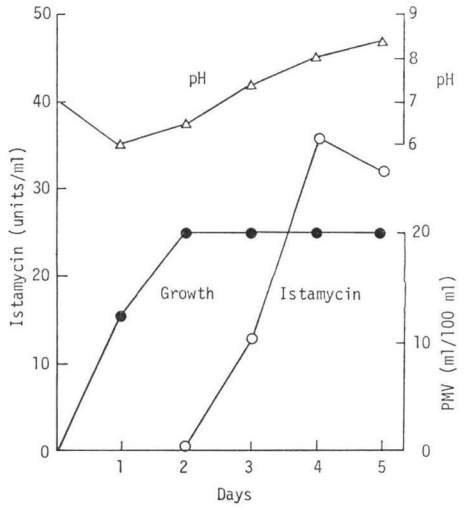
* SBM=Soy bean meal, Starch=Potato starch.
** Incubation days.
*** μg/ml as istamycin A.

Table 4. Effect of palmitate on istamycin production.

Sodium palmitate (%)	Istamycin (μg/ml)		
	72 hrs.	84 hrs.	96 hrs.
0	tr	9	16
0.10	13	45	41
0.15	22	41	41
0.20	18	52	41
0.25	11	35	35
0.30	4	15	25

Basal medium: Potato starch 2.0%, soy bean meal 2.0%, glucose 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% and NaCl 0.3%.

Fig. 1. Time course of istamycin production by *Streptomyces tenjimariensis* SS-939.
Packed mycelium volume (PMV) was measured after centrifugation (3,000 rpm for 10 minutes).



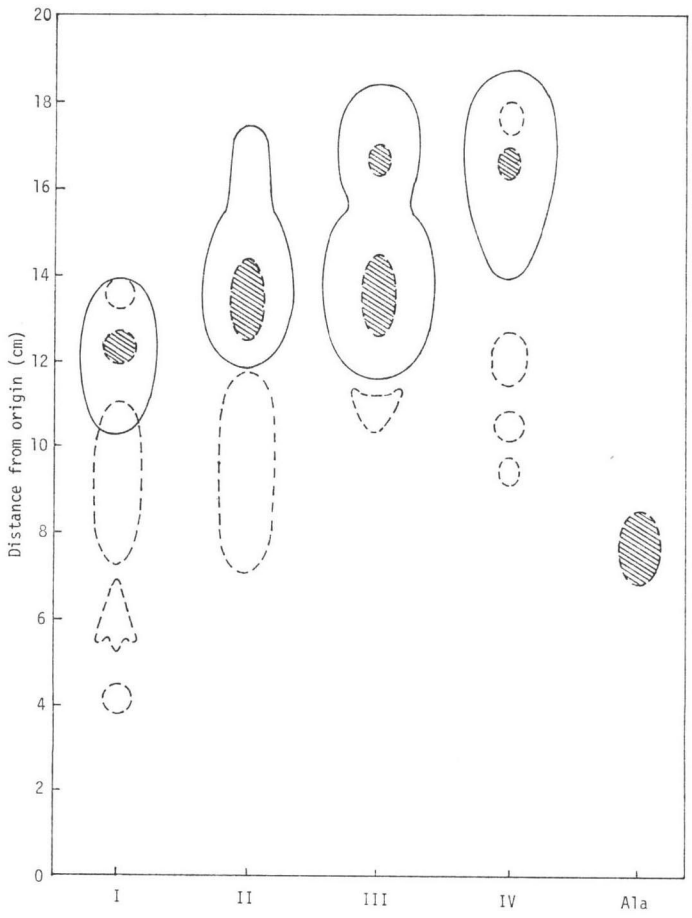
viscous gel and istamycin production was markedly lowered.

In a previous paper[3], aliphatic fatty acids such as palmitic and stearic acids, especially palmitate, were found to stimulate istamycin production. Table 4 shows the effect of palmitate concentration. When the medium was not supplemented with sodium palmitate, the istamycin yield was as low as 16 μg/ml after 4 days of incubation. Addition of palmitate ranging from 0.1% to 0.25% enhanced istamycin production more than two-fold.

Metal ions (Na^+, Co^+, Mg^{++}, Ca^{++}, Zn^{++} and Mn^{++}) were also examined for their effect on istamycin production at a concentrations of 0.1% of their sulfates or chlorides, but no significant effects were observed (data not shown).

Thus the basic medium described in Materials and Methods proved favorable for istamycin production. Fig. 1 shows the time course of istamycin production by *S. tenjimariensis* SS-939 in the basic medium. The organism grew well and reached the stationary phase of growth after 2 days of incubation. Istamycin production was not initiated until the culture reached stationary phase, and the maximum accumulation of istamycin was observed after 4 days of incubation. The pH of the medium during incubation went down to 6, then began to rise gradually and reached about 8 when the maximum amount of istamycin was accumulated.

Fig. 2. High voltage paper electrophoresis of culture filtrate (eluate from Amberlite CG50 (NH_4^+)).
Solid circle: Bioautogram
Dotted circle (with oblique line): Ninhydrin positive (strong)



Chemical and Biological Properties of Istamycins A and B

Supplementing the information presented in our first paper[1] describing chemical and biological properties of istamycins A and B, some additional data will be presented.

Fig. 2 shows bioautogram and ninhydrin reactions following high-voltage paper electrophoresis of istamycins. Istamycins were adsorbed on a column of Amberlite CG-50 (NH_4^+), eluted with aqueous ammonia (1 N) and collected in small fractions according to the method described in a previous paper[1]. Active fractions were roughly divided into 4 groups and examined by high-voltage paper electrophoresis. Two major active spots, which moved to the cathode with Rm (mobility relative to alanine) 2.15 and 1.75 were detected. Istamycins A and B, the structures of which have already been reported in our first paper[1], were present in the Rm 2.15 spot. The other istamycins in the Rm 1.75 active spot are now being analyzed for their structures and biological activities. Istamycin consists of more than 20 components, judging from data obtained so far.

Since istamycins have structures similar to aminoglycoside antibiotics such as fortimicins, it was not possible to distinguish istamycins from fortimicin A by high-voltage paper electrophoresis. How-

Table 5. Thin-layer chromatography of istamycins.

Antibiotics	Rf			
	Silica gel		Cellulose	
	Sol. A	Sol. B	Sol. A	Sol. C
Istamycin A	0.30	0.17	0.93	0.21
Istamycin B	0.15	0.08	0.83	0.25
Istamycin A_0	0.46	0.18	0.98	0.31
Istamycin B_0	0.18	0.14	0.83	0.30
Fortimicin A			—	0.12
Sporaricin A			0.74	—

Sol. A: $CHCl_3$-MeOH-17% NH_4OH, 2 : 1 : 1 (v/v, lower layer).
Sol. B: $CHCl_3$-MeOH-17% NH_4OH, 1 : 8 : 1 (v/v).
Sol. C: BuOH-Pyridine-AcOH-H_2O, 6 : 4 : 2 : 4 (v/v).
Detection: ninhydrin.

ever, istamycins A and B were distinguished from sporaricin A and fortimicin A on cellulose TLC as shown in Table 5.

In Table 6, antibiotic activity spectra of istamycins A and B were compared with those of fortimicin A and sporaricin A. Istamycins A and B are as active as fortimicin A and sporaricin A against Gram-positive and Gram-negative bacteria including aminoglycoside resistant strains. Istamycin B is more active than istamycin A and also shows a somewhat stronger activity against AAC(3)-I-producing organisms than fortimicin A and sporaricin A; all the antibiotics are substantially inactive against the AAC(3)-I-producing *Escherichia coli* strain.

Table 6. Antimicrobial spectrum of istamycins A and B.

Organisms	Enzyme	Minimum inhibitory concentrations (μg/ml)			
		Istamycin A	Istamycin B	Fortimicin A	Sporaricin A
Staphylococcus aureus FDA 209P		1.56	0.78	3.13	0.78
Staphylococcus aureus Smith		0.39	<0.10	0.39	<0.10
Staphylococcus aureus ApO 1	ANT (4′)	1.56	1.56	3.13	0.78
Staphylococcus epidermidis 109	ANT (4′)	1.56	1.56	3.13	0.78
Bacillus subtilis PCI 219		0.39	<0.10	0.39	0.2
Mycobacterium smegmatis ATCC 607		1.56	0.78	NT	NT
Escherichia coli NIHJ		3.13	1.56	6.25	1.56
Escherichia coli K-12		3.13	1.56	6.25	1.56
Escherichia coli K-12 R 5	AAC (6′)	6.25	6.25	25	3.13
Escherichia coli K-12 ML 1629	APH (3′)-I	3.13	3.13	12.5	3.13
Escherichia coli K-12 LA 290 R 55	ANT (2″)	6.25	3.13	12.5	3.13
Escherichia coli JR 66/W 677	APH (3′)-II ANT (2″)	6.25	6.25	12.5	6.25
Escherichia coli K-12 C 600 R 135	AAC (3)-I	>50	25	>50	>50
Escherichia coli JR 225	AAC (3)-IV	3.13	1.56	6.25	3.13
Klebsiella pneumoniae PCI 602		6.25	3.13	6.25	1.56
Shigella dysenteriae JS 11910		12.5	6.25	12.5	6.25
Shigella flexneri 4b JS 11811		12.5	6.25	12.5	6.25
Salmonella typhi T 63		1.56	12.5	25	12.5
Salmonella enteritidis 1891		3.13	3.13	6.25	1.56
Proteus vulgaris OX 19		1.56	0.78	1.56	0.78
Serratia marcescens		25	12.5	50	6.25
Pseudomonas aeruginosa A 3		>50	12.5	25	12.5
Pseudomonas aeruginosa No. 12		>50	>25	>50	>50

NT: not tested.

Discussion

Streptomyces tenjimariensis SS-939 produced about 30~40 μg/ml of istamycins in a starch-soy bean meal medium. Istamycin production was markedly inhibited when starch was replaced with glucose, maltose, glycerol, inositol or glucosamine. This inhibition might be due to carbon catabolite inhibition and/or repression common in many antibiotic fermentations[5]. It was somewhat surprising that inositol showed no effect. Deoxy-*scyllo*-inositol is recognized as an intermediate in DOS synthesis, which suggests that inositol might be incorporated into the istamycin molecule as part of the aminocyclitol moiety[6].

Several nitrogen sources such as yeast extract, peptone and casamino acid added to the medium in place of soy bean meal also showed a marked inhibition of istamycin production, although the organism showed good growth in all the media. This is probably partly due to nitrogen catabolite regulation[5,7] and partly to hydrolysis of the istamycins formed because of the rapid rise of pH to a value above 9 in those media, reducing measured istamycin activity.

Palmitate was the only factor so far examined stimulating istamycin production. It was reported that neomycin production was restored in an *S. fradiae* crypto-producer strain when oleic and palmitic acids were added, bringing about an alteration of membrane fatty acid composition[8,9]. Similarly, stimulation of istamycin production by palmitate may be also connected to the alteration of membrane permeability.

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