A NEW ANTIBIOTIC, IKARUGAMYCIN

KAZUYOSHI JOMON, YOSHIO KURODA,
MUTSUYA AJISAKA and HEIICHI SAKAI

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.
Higashiyodogawa-ku, Osaka, Japan

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A protozoan *Tetrahymena pyriformis* W as a test organism can be useful in the preliminary screening for antiprotozoals, especially antitrichomonas agents. By this screening method, ikarugamycin, a new antibiotic with specific antiprotozoal activity was found in the culture of *Streptomyces* sp. No. 8603 isolated from a soil sample. The morphological characteristics of *Streptomyces* sp. No. 8603 include formation of gray aerial mycelium, dark brown growth and aerial hyphae forming a long spiral. Strain No. 8603, belonging to a chromogenic type, was identified as a variety of *Streptomyces phaeochromogenes* and given the name *Streptomyces phaeochromogenes* var. *ikaruganensis* SAKAI.

Ikarugamycin was isolated as white crystalline needles, decomposing at 252~255°C, and exhibiting optical rotation $[\alpha]_D^{20}+360°$ (*c* 1.10, dimethylformamide). Its molecular formula $C_{29}H_{38}O_4N_2$ was given by elementary and mass spectrum analyses. The ultraviolet absorption gave two maximal peaks at 220 mμ and 325 mμ in methanol. Ikarugamycin showed strong antiprotozoal activities: MIC 0.3~1.25 mcg/ml against *Trichomonas vaginalis*, MIC 1.0 mcg/ml against *Tetrahymena pyriformis* W and MIC 2~10 mcg/ml against *Entamoeba histolytica*. The median lethal dose of ikarugamycin was 6 mg/kg, determined in mice by intraperitoneal administration. Hexahydro-ikarugamycin was obtained by catalytic hydrogenation of ikarugamycin and its physicochemical and biological properties were investigated. It is similarly active against *T. vaginalis* as is the antitrichomonas agent, azalomycin F and its acute toxicity (LD_{50} 300 mg/kg, ip, in mice) is less than that of ikarugamycin and azalomycin F.

In our laboratories a screening method using *Tetrahymena pyriformis* W as an indicator organism for antiprotozoal products was devised. As a result of this screening program, *Streptomyces* No. 8603 was found to produce a new antibiotic with strong antiprotozoal activity which was designated as ikarugamycin.

This paper deals with the screening method as well as taxonomic studies on the producing organism, the production and isolation of ikarugamycin and the physicochemical and biological properties of ikarugamycin. In addition, a derivative of ikarugamycin with low toxicity, hexahydro-ikarugamycin is presented.

Screening Method

A specific screening method for antiprotozoal substances produced by microorganisms has been desired. Various protozoa have been used as model organisms for

screening of biologically active substances[1]. *Chlorella* sp. have been used in the screening for plant growth-accelerating substances[2] and antitumor agents[3], *Euglena gracilis* used for antiprotozoals[4,5,6,7] and plant regulators[8], and *Turbatrix aceti* for antibiotics with antihelminthic activity[4,9,10].

NEMEC *et al*[11] have reported a new method for the primary screening of antibiotic with antiprotozoal activity using *Trypanosoma cruzi* as the test organism. Moreover, for screening antiprotozoal antibiotics, they[5,6,7] examined the cultures of fungi using six kinds of protozoa including *Tetrahymena pyriformis* and ascertained the activity by the loss of motility of the pratozoa. In addition, a screening model for the search of new trichomonacides using *Ochromonas malhamensis* as an indicator organisnism has been established by THIEMANN *et al.*[12] Two protozoa, *Tetrahymena* and *Ochromonas*, were used to study the mode of action and side effects of antihistamines[13]: The mode of action was studied by inhibition analysis and the side effects by motility tests.

With much information from the reports described above, the screening method for trichomonacide using *Tetrahymena pyriformis* as the test organism was devised. For routine screening for trichomonacides, we used a ciliated protozoan, *T. pyriformis* W, as an intermediate indicator organism for the following reasons:

1) This organism is non-pathogenic for man, and in addition is suitable for microscopic observation since its cell size is comparatively large (20~30 μ).

2) By motility observations, this organism is resistant to most of the toxic antibiotics frequently found in the culture broth of streptomycete (Table 1).

The cells of *T. pyriformis* W were grown at 25°C and maintained by transfer every week in 10 ml of broth which contained 1.0 % of Proteose-peptone (Difco) and 0.1 % of yeast extract (Difco) in 50-ml Erlenmyer flask. For screening the organism, was cultivated in the medium at 25°C for 15 hours. A half ml aliquot of *T. pyriformis* W was added to 0.5 ml of each sample and incubated at room temperature for 20~60 minutes. After incubation, morphological changes and motility of the organism were observed microscopically. A progressive increase in the concentration of active substance caused the cells of *Tetrahymena* to become rounded, vacuolated and swollen. This was followed eventually by lysis of the cell membrane.

The activity of the test materials was evaluated by degree of dilution index and changes in the morphological type and motility of the organism. In our screening program, the degree of inhibitory effect on the morphology and motility was expressed as follows:

3+: most organisms exploded or all organisms rounded.

Table 1. The effect of known antibiotics on *T. pyriformis* W*

Antibiotics	Concentration (mcg/ml)			
	50	25	10	5
Actinomycin	—	—	—	—
Anthelmycin	—	—	—	—
Anthramycin	2+	—	—	—
Antimycin	—	—	—	—
Aureolic acid	—	—	—	—
Azalomycin F	3+	3+	3+	+
Blasticidin S	—	—	—	—
Blastomycin	—	—	—	—
Dextrochrysin	2+	—	—	—
Dihydroteleocidin B	—	—	—	—
Echinomycin	—	—	—	—
Mitomycin	—	—	—	—
Tomaymycin	—	—	—	—
Toyocamycin	—	—	—	—

* Activity against protozoon after 30 minutes. For explanation of abbreviations see text.

2+: most organisms showed sluggish motility.

1+: 50 % of organisms showed motility, 50 % rounded.

−: most organisms were actively motile.

Table 2. The effects of aqueous organic solvents on *T. pyriformis* W*

Organic solvents	Concentration (%)					
	12.5	6.25	3.13	1.56	0.78	0.38
Methanol	3+	+	−	−	−	−
Ethanol	3+	3+	+	−	−	−
Acetone	3+	+	−	−	−	−
Dioxane	3+	3+	3+	+	−	−
Tetrahydrofuran	3+	3+	3+	2+	−	−
Dimethylformamide	3+	3+	−	−	−	−
Pyridine	3+	3+	3+	3+	2+	−

* Activity against protozoon after 30 minutes. For explanation of abbreviations see text.

Actinomycetes isolated from soils were cultured in adequate media at 30°C for 4 days on a reciprocal shaker. Culture broths were filtered and mycelia were extracted with dimethylformamide, which was proved to be non-toxic against the test organism according to our experiments (Table 2). Filtrates and mycelial extracts were diluted with water by the serial two-fold dilution method and used for the antitetrahymena assay as described above.

Taxonomic Studies of the Producing Organism

Ikarugamycin is produced by a strain, No. 8603 isolated from a soil sample collected in Ikarugamachi, Nara Prefecture, Japan. According to the taxonomical studies described below this organism was identified with a new variant of *Streptomyces phaeochromogenes* and designated as *Streptomyces phaeochromogenes* var. *ikaruganensis* SAKAI.

The morphology of the culture on CZAPEK's agar was microscopically observed (Plates 1 and 2). Aerial hyphae of this culture repeatedly branch, generally forming long spirals. The surface of spore is smooth. No. 8603 strain generally grows well at 25~30°C at pH 6~8.

The experiments to determine the following cultural characteristics were carried out at 30°C for 10~14 days. The gelatin stab culture was observed after incubation at room temperature for 20 days.

CZAPEK's agar: Scant growth, greenish black; aerial mycelium, powdery, white with black spots; brownish black soluble pigment.

Starch ammonium agar: Growth, little colorless colonies with black spots; no aerial mycelium; no soluble pigment. Starch is hydrolyzed strongly.

Glucose asparagine agar: Growth, little colonies, dark brown; aerial mycelium, abundant, powdery light gray; brown soluble pigment.

Calcium malate agar: Good growth, dark brown; aerial mycelium, powdery, abundant, dark gray; brown soluble pigment.

Tyrosine agar: Scant growth; no aerial mycelium; no soluble pigment.

Bouillon agar: Good growth, raised, reddish brown; aerial mycelium, powdery, gray; brown soluble pigment.

Plate 1.

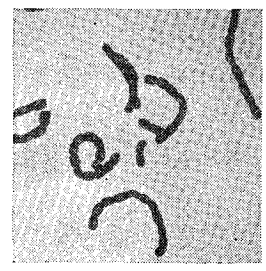
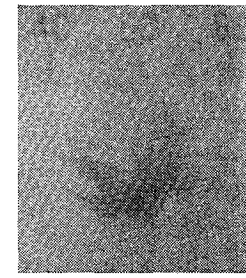


Plate 2.



BENNETT's agar: Growth, little colonies, black; aerial mycelium, powdery, gray; brownish black soluble pigment.

BENNETT's agar (37°C): Good growth wrinkled, pale cream; no aerial mycelium; no soluble pigment.

Glucose bouillon broth: Good growth, thick colonies grown on surface; aerial mycelium, powdery, gray; brownish black soluble pigment.

Table 3. Utilization of carbon sources

	Utilization		Utilization
Glucose	+	Sucrose	±
Rhamnose	+	Lactose	±
Salicin	+	Trehalose	±
Fructose	±	Raffinose	±
Xylose	±	Inositol	±
Arabinose	±	Mannitol	±
Mannose	±		

+ : Moderately good growth ± : Faint growth

Glucose-CZAPEK's solution: Good growth, brown colonies grown on surface; aerial mycelium, powdery, gray; greenish brown soluble pigment. Nitrites not produced from nitrate.

Milk: Dark brown ring; no aerial mycelium; greenish brown soluble pigment. Peptonized with coagulation.

Gelatin stab (20°C, 20 days): Good growth, brownish black; no aerial mycelium; brownish black soluble pigment. Liquefaction.

Potato plug: Good growth, raised, brownish black; aerial mycelium, powdery, gray; black soluble pigment (no diffusion).

Cellulose: Scant growth, dark green; aerial mycelium, powdery, gray; no soluble pigment. Cellulose is not hydrolyzed.

The carbon utilization of No. 8603 strain was examined according to the method described by PRIDHAM and GOTTLIEB[14] with results as shown in Table 3.

The distinctive characters of No. 8603 strain would be as follows:

1) The strain shows typically chromogenic character and produces brownish black soluble pigments in the media containing protein and in synthetic media.

2) The gray color is typical of the aerial mycelia. Microscopically observed, its aerial hyphae form long spirals.

3) Amylolytic action is strong, moreover, proteolytic activities on gelatin or milk are both strong.

The taxonomic keys of WAKSMAN's The Actinomycetes Vol. 2[15] and Reports of International Streptomyces Project[16] were used to compare the culture with recognized species of the genus *Streptomyces*.

Among known species of *Streptomyces*, *S. phaeochromogenes*[17], *S. melanogenes*[18] and *S. noboritoensis*[19] were found to be closely related to this organism. However, detailed comparison shows some differences among them. Namely, *S. melanogenes* forms aerial mycelium with no spiral and yellowish brown~yellowish green soluble pigments in synthetic media. *S. noboritoensis* forms aerial mycelium with no regular spiral, light soluble pigments in synthetic media and exhibits poor activities in both amylolytic and proteolytic characters. The characteristics of the strain No. 8603 described above place this organism within the *Streptomyces phaeochromogenes* group as defined by CONN, 1917[17].

Characteristics which distinguish No. 8603 strain from standard strains of *S. phaeochromogenes* are:

1) Formation of brownish black soluble pigments.
2) Ability to produce a new antibiotic, ikarugamycin.

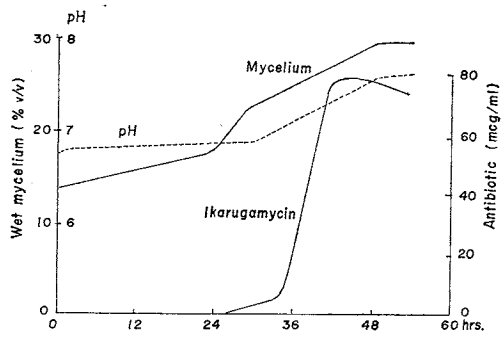
From these considerations, No. 8603 strain is reasonably recognized as a variant of *S. phaeochromogenes* and designated as *Streptomyces phaeochromogenes* var. *ikarugaensis* SAKAI.

Fermentation and Isolation of Ikarugamycin

Slant cultures of the strain *S. phaeochromogenes* var. *ikaruganensis*, No. 8603 grown for at least 7 days at 28°C on BENNETT's agar was inoculated into 100 ml of the medium consisting of the following ingredients: starch 2%, Pharmamedia (Trader Oil Mill Co., Texas, U.S.A.) 2%; corn steep liquor 1% and $CaCO_3$ 0.3% (initial pH 6.2). After 24 hours of incubation at 30°C on a reciprocal shaker, a 200-liter stainless steel fermentor containing 60 liters of the same medium was inoculated with 5% (v/v) of the shaker cultures and incubated at 30°C as a seed tank, agitated at 250 rev./min. and aerated with 0.5 v/v/min. Then the broth in the seed tank after 24 hours was transferred to 600 liters of the same medium in a 1,000-liter stainless steel tank. Fermentation was run at 30°C, agitated at 183 rev./min. and aerated with 0.5 v/v/min. for 48 hours. A typical fermentation process is shown in Fig. 1.

During the fermentation and isolation process, the antiprotozoal activity was assayed by the evaluation of degree of inhibitory effect on the motility of *T. pyriformis* W.

Fig. 1. Fermentation of ikarugamycin
660 liters of medium were placed in a 1,000-liter stainless steel fermentor. Aeration rate: 600 liters/min. Agitation rate: 180 r.p.m.



Ikarugamycin was produced in the filtrate, but mostly in the mycelium. The mycelial cake was washed with acetone to remove the colored impurities and extracted twice with a mixture of chloroform and methanol (1:1). The extract was concentrated *in vacuo*. The aqueous concentrate was dissolved in 10% aqueous tetrahydrofuran and treated with two volumes of acidic water (pH 3~4, 2 N HCl) to precipitate the active factor. The crude precipitate was washed with acetone and dried *in vacuo*. The dry active powder was dissolved in chloroform or chloroform-methanol and crystallized. White crystalline needles were obtained by recrystallization from chloroform-methanol or chloroform-acetone.

Physicochemical Properties of Ikarugamycin

Ikarugamycin is a white crystalline needles which melt at 252~255°C with decomposition. It behaved as a single spot on thin-layer chromatography, using Silicic acid (Mallinckrodt); Rf 0.70~0.80, developed by a solvent mixture of chloroform and methanol (9:1) and using Silica gel G (Merck); Rf 0.60~0.70, *n*-butanol-acetic acid-water (4:1:1).

Ikarugamycin is poorly soluble in the usual organic solvents. It is soluble at about 10 mg/ml in the following solvents (at 50°C): N,N-dimethyl-acetamide, N,N-dimethyl-formamide, hexamethyl-phosphoramide and aqueous tetrahydrofuran. It is slightly soluble in chloroform, dichloroethane, pyridine, acetone and alcohols, but insoluble in *n*-hexane, petroleum ether, diethyl ether and water.

Fig. 2. Ultraviolet spectrum of ikarugamycin

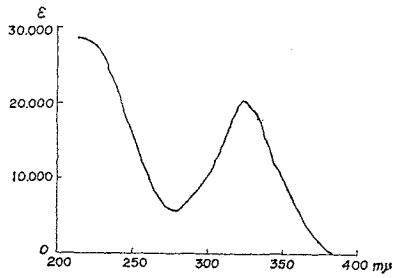
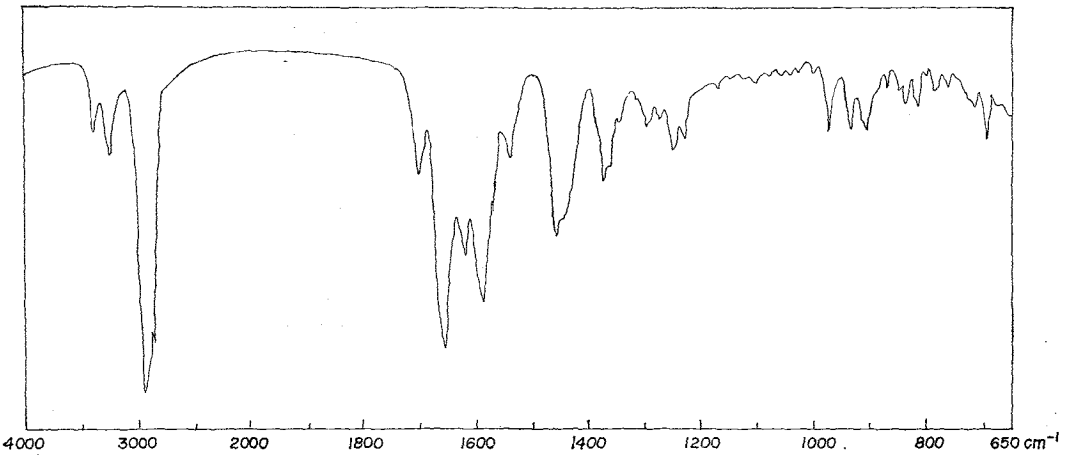


Fig. 3. Infrared absorption spectrum of ikarugamycin (Nujol)



The ultraviolet absorption spectrum of ikarugamycin is shown in Fig. 2, with peaks at 220 mμ (ε 28,700) and 325 mμ (ε 20,000) in methanol. The infrared absorption spectrum in Nujol is shown in Fig. 3, with absorptions at the following frequencies; 3390, 3250, 2920, 2860, 1750, 1658, 1623, 1590, 1545, 1460, 1378, 1345, 1295, 1275, 1252, 1232, 978, 940, 909, 870, 845, 823 and 703 cm^{-1}.

It shows strong optical rotation, $[\alpha]_D^{20}+360°$ (*c* 1.10, dimethylformamide). It is weakly acidic; pKá 5.57 (50 % aqueous tetrahydrofuran).

Elementary analysis of ikarugamycin gave: C 72.35, H 8.09, N 5.87, O 13.84 (%). Calcd. for $C_{29}H_{38}O_4N_2$ (M.W. 478): C 72.77, H 8.00, N 5.85, O 13.37 (%). The emprical formula $C_{29}H_{38}O_4N_2$ is established by the parent peak in the mass spectrum at *m/e* 478 and the elementary analysis.

Ikarugamycin is colored with iodine vapor and decolorizes potassium permanganate. It shows a positive reaction in ferric chloride test and negative ninhydrin, EHRLICH's and DRAGENDORFF's tests. Ikarugamycin is stable in neutral or alkaline solution at room temperature, but labile in acidic solution. The crystalline powder is stable for 2 weeks at 70°C, after 4 weeks at 60°C and after 2 months at 45°C.

Biological Properties of Ikarugamycin

The antimicrobial spectra of ikarugamycin shown in Table 4 were determined by the agar dilution streak method, unless otherwise indicated.

Table 4. Antimicrobial spectra of ikarugamycin

Organisms	MIC (mcg/ml)
Trichomonas vaginalis 4 FM*	0.5**
〃 〃 1*	1.25**
〃 〃 9*	0.625**
〃 〃 11*	0.313**
Tetrahymena pyriformis W	1.0**
〃 〃	0.1～0.2***
Entamoeba histolytica	2～10
Trypanosoma cruzi (Wellcome strain)	negative
Coccidiodes (chicken)	negative
Staphylococcus aureus Newman	6.25
〃 〃 Terajima	12.5
〃 〃 FDA 209 P	12.5
Bacillus subtilis ATCC 6633	6.25
〃 〃 PCI-219	6.25
Sarcina lutea PCI-1001	50
Escherichia coli NIHJ	>100
Klebsiella pneumoniae ST-101	>100
Salmonella typhosa T-289	>100
〃 *enteritidis*	>100
Shigella flexneri	>100
〃 *sonnei*	>100
Proteus vulgaris IAM 1025	>100
Pseudomonas aeruginosa IAM 1095	100
Candida albicans YU 1200	>100
Saccharomyces cerevisiae 11299	>100
Penicillium chrysogenum IAM 7326	>100
Diplococcus pneumoniae	100
Streptococcus pyogenes S-23	100
〃 *faecalis*	>100

* clinical isolates
** broth dilution method
*** inhibitory effect on the motility of the organism

Table 5. Amoebicidal activity of conventional antiamoebic drugs and antibiotics *in vitro*.

Drug	Ameba strain	No. of exp.	Effective drug concentratilon (mcg/m)
Emetine hydrochloride	YS 14	2	100
	YS 15	2	200
	NAMRU II	2	100
	YS 24	2	50
	YS 25	2	50
Carbarsone	YS 14	2	50
	YS 15	2	50
	NAMRU II	3	50
	YS 24	3	100
	YS 25	3	100
Atabrine	YS 14	2	500
	YS 15	2	250
	NAMRU II	2	1,000
	YS 24	2	500
	YS 25	2	500
Tetracycline	YS 14	1	200
	YS 15	1	200
	NAMRU II	2	125
	YS 24	2	200
	YS 25	2	200
Niridazole	YS 14	2	0.2
	YS 15	1	1
	NAMUR II	3	2
	YS 24	4	2
	YS 25	3	2
Metronidazole	YS 14	1	10
	YS 15	1	10
	NAMRU II	2	10
	YS 24	3	20
	YS 25	2	20
Ikarugamycin	YS 14	1	10
	YS 15	1	10
	NAMRU II	2	10
	YS 24	3	2
	YS 25	2	2.5

Ikarugamycin has strong activity against protozoa: minimum inhibitory concentration of 0.3～1.25 mcg/ml against *Trichomonas vaginalis*, 1.0 mcg/ml against *Tetrahymena pyriformis* W and 2～10 mcg/ml against *Entamoeba histolytica*. Activity against *E. histolytica* (*in vitro*), shown in Table 5, was kindly determined by Dr. KEE MOK CHO *et al.*, Yonsei University College of Medicine, Seoul, Korea.

In the experiment with ikarugamycin, cyst-born amoeba (YS 14, YS 15 and NAMARU -strain) were less sensitive than the trophozoite-born amoeba (YS 24 and YS 25 strains) in contrast with nitridazole and metronidazole. Ikarugamycin showed excellent antiamoebic activities *in vitro*.

Ikarugamycin is also active against some Gram-positive bacteria, but not active against yeast and fungi. The activities of ikarugamycin against Gram-positive bacteria cannot be detected by paper disc or cylinder method because of its low diffusion in agar media.

Ikarugamycin is quite toxic (LD_{50} 6 mg/kg, ip, in mice) and causes hemolysis at low concentrations (3.5 mcg/ml) in rabbit blood.

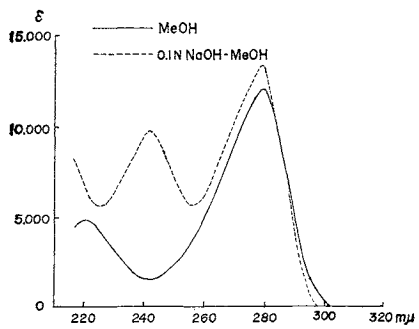
Hexahydro-ikarugamycin

Looking for new derivatives with less toxicity, we have made some trials to transform the structure of ikarugamycin. As a result of those experiments hexahydro-ikarugamycin was successfully prepared by catalytic hydrogenation on PtO_2 in ethanol solution. It is recrystallized from chloroform to be white crystalline needles, melting at 243~245°C with decomposition.

Elementary analysis of hexahydro-ikarugamycin gave: C 71.68, H 9.14, N 5.56, O 13.56 (%). Calcd. for $C_{29}H_{44}O_4N_2$ (M.W. 484): C 71.86, H 9.15, N 5.78, O 13.21 (%). The molecular formula of this compound is confirmed to be $C_{29}H_{44}O_4N_2$ by the parent peak in the mass spectrum at *m/e* 484, the absorption of hydrogen and the elementary analysis.

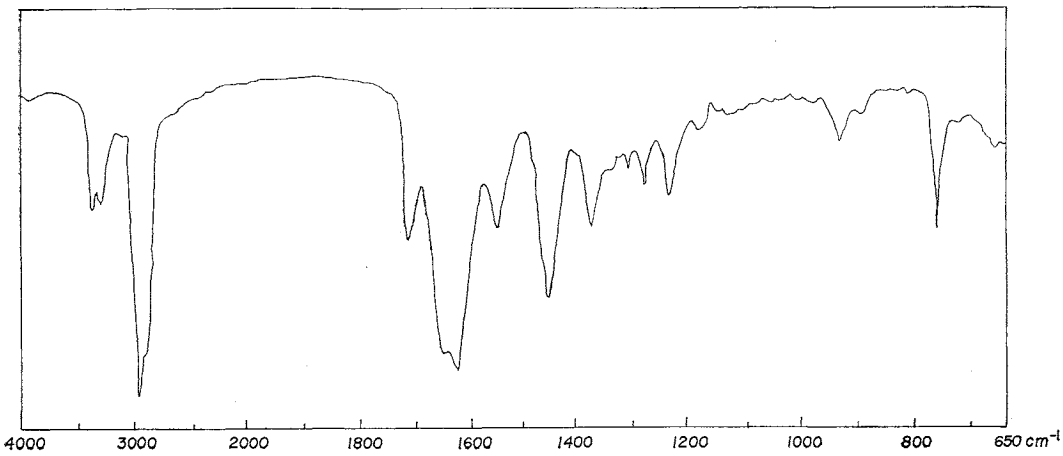
Its solubility is similar to that of ikarugamycin. The ultraviolet absorption spectra are shown in Fig. 4, indicating peaks at 220 mμ (ε 5,000) and 280 mμ (ε 12,400) in methanol, and at 243 mμ (ε 10,200) and 280 mμ (ε 13,600) in 0.1 N NaOH-methanol, respectively.

Fig. 4. Ultraviolet spectra of hexahydro-ikarugamycin



The infrared absorption spectrum in Nujol is indicated in Fig. 5. It shows optical rotation $[\alpha]_D^{20}$ +140° (*c* 0.6, dimethylformamide). It is weakly acidic, pKá 5.05 (in 67 % ethanol). As

Fig. 5. Infrared absorption spectrum of hexahydro-ikarugamycin (Nujol)



shown in Table 6, hexahydro-ikarugamycin shows the similar activity against *T. vaginalis* to that of ikarugamycin and azalomycin F[20], but its acute toxicity is significantly less than that of ikarugamycin and azalomycin F.

Table 6. Comparison of biological characteristics

		Hexahydro-ikarugamycin	Ikarugamycin	Azalomycin F[20]
Antimicrobial activity		MIC (mcg/ml)		
Trichomonas vaginalis		6.25	0.3~1.25	6
〃 *foetus*		6.25	0.5	
Tetrahymena pyriformis W		8	0.1~0.2	
Penicillium chrysogenum		>100	>100	1.56~3.12
Candida albicans		>100	>100	1.56
Staph. aureus FDA 209 P		50	12.5	6.25
Bacillus subtilis PCI-219		50	6.25	3.12
Escherichia coli		>100	>100	>100
Pseudomonas aeruginosa		>100	100	>100
Hemolysis (mcg/ml)		25	3.5	
Acute toxicity LD_{50} in mice (mg/kg)	po	>800	>500	
	sc	>800	>250	
	ip	300	6	26
	iv	30	2	

Discussion

Our screening method using *T. pyriformis* W is characterized by the microscopical observation of motility and morphological changes of the organism after short incubation (20~60 minutes). This method seems to be useful for the screening of new antitrichomonacides among microbial products.

Ikarugamycin and its new derivative hexahydro-ikarugamycin can be differentiated from known antibiotics as follows:

The antibiotics with two maxima within the same range of wavelength of ikarugamycins are: althiomycin[21], antimycin[22], blastmycin[23], methymycin[24], and vulgarin[25]. But, elementary analysis, optical rotation, melting point and infrared spectrum differentiate ikarugamycins from these antibiotics. Besides them, thaimycin C[26], which exhibits an antiprotozoal activity, resembles ikarugamycins in ultraviolet spectrum, melting point, solubility and stability. However, thaimycin C differs from the ikarugamycins in elementary analysis, optical rotation and infrared spectrum.

On the basis of physicochemical properties of ikarugamycins, both ikarugamycin and hexahydro-ikarugamycin are recognized to be new antibiotics.

Acknowledgement

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