KARABEMYCIN, A NEW ANTIMETABOLITE OF GLUTAMINE PRODUCED BY A STRAIN OF STREPTOMYCETE

SATOSHI ŌMURA, AKIRA NAKAGAWA, HIROSHI AOYAMA and YUZURU IWAI

Kitasato University and The Kitasato Institute, Minato-ku, Tokyo 108, Japan

MASAO KUWAHARA and TAKASHI FURUSATO

Nissan Chemical Industries Ltd., 1470 Shiraoka, Minamisaitama-gun, Saitama, Japan

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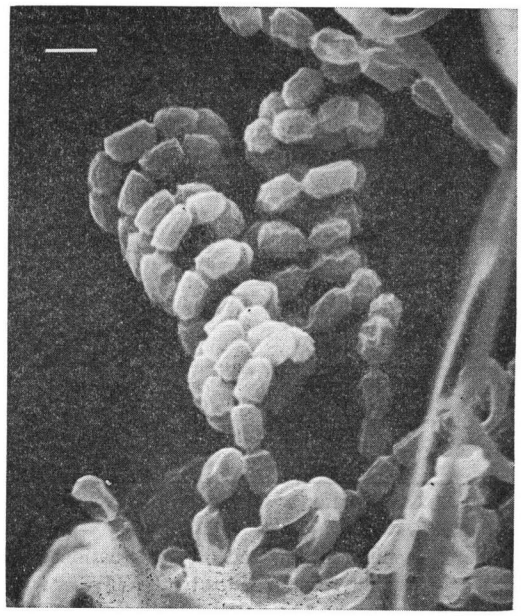
Karabemycin, a new peptide antibiotic, was isolated from the culture filtrate of *Streptomyces* sp. AM-6424, a soil isolate. The molecular formula of the antibiotic was determined as $C_{15}H_{24}N_4O_6$ on the basis of elemental analysis, FD-mass spectrum and ^1H and ^{13}C NMR. Acid hydrolysate of karabemycin contains one mol each of valine, alanine and an unknown amino acid.

The antibiotic is active against Gram-positive and -negative bacteria on a synthetic medium. The inhibitory activity is reversed by L-glutamine and L-glutamic acid.

In the course of screening for new antibiotics of actinomycetes origin, we found that strain AM-6424 isolated from a soil sample collected at Karabe, Narita City, Chiba Prefecture, Japan, produces a new peptide antibiotic which has been designated karabemycin. The antibiotic is active against some bacteria such as *Bacillus subtilis* PCI 219 and *Escherichia coli* IFO 12734 on a synthetic medium, and its activity is reversed by both L-glutamine and L-glutamic acid.

The present paper deals with the taxonomy of strain AM-6424 and the production, isolation, and biological and physicochemical properties of karabemycin.

Plate 1. Scanning electron micrograph of aerial mycelia of *Streptomyces* sp. AM-6424.
Bar represents 1 μm.



Taxonomy of the Producing Organism

Morphology

The vegetative mycelium grows abundantly on both synthetic and complex agar media, and does not have septa. Velvety and powdery aerial mycelia were well produced on various agar media. Plate 1 shows scanning electron micrograph of aerial mycelium grown on inorganic salts - starch agar. The sporophores are of the

Table 1. Cultural characteristics of strain AM-6424.

Medium	Cultural characteristics
Yeast extract - malt extract agar*	G: Good, amber (3pc)
	R: Orange rust (4pe)
	AM: Moderate, light ivory (2ca)
	SP: Bright yellow (3na)
Oatmeal agar*	G: Good, mustard gold (2pe)
	R: Amber (3pc)
	AM: Abundant, cottony, covert tan (2ge)
	SP: Gold (21c)
Inorganic salts - starch agar*	G: Good, mustard gold (2ne)
	R: Gold (21c) and amber (3pe)
	AM: Abundant, velvety, white (a)
	SP: Light gold (1½kb)
Glycerol - asparagine agar*	G: Good, squash yellow (2ie)
	R: Golden yellow (2ob)
	AM: Abundant, velvety, light ivory (2ca)
	SP: Golden yellow (2kb)
Glucose - asparagine agar	G: Good, gold (2lc)
	R: Amber (3pc)
	AM: Abundant, velvety, white (a)
	SP: Sunlight yellow (1½ia)
Peptone - yeast extract - iron agar*	G: Good, beige brown (3ig)
	R: Mustard tan (2lg)
	AM: None
	SP: Poor, light mustard tan (2ie)
Tyrosine agar*	G: Good, golden brown (3pg)
	R: Golden brown (3pg)
	AM: Abundant, velvety, pale yellow (1ca)
	SP: Squash yellow (2ia)
Sucrose - nitrate agar**	G: Moderate, ivory (2db)
	R: Ivory (2db)
	AM: Poor, light ivory (2ca)
	SP: None
Glucose - nitrate agar**	G: Good, light amber (3ic)
	R: Amber (3nc)
	AM: Abundant, velvety, ivory (2db)
	SP: Bright yellow
Glycerol - calcium malate agar**	G: Good, gold (1½nc)
	R: Mustard gold (2nc)
	AM: Poor, parchment (1½db)
	SP: Light olive gray (1½ge)
Glucose - peptone agar**	G: Good, dark luggage tan (4pg)
	R: Orange rust (4pe)
	AM: Abundant, velvety, white (a)
	SP: Oak brown (4pi)
Nutrient agar**	G: Moderate, light mustard tan (2ie)
	R: Mustard brown (2pi)
	AM: Poor, white (a)
	SP: Bamboo (2gc)

Abbreviation: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

* Medium recommended by International Streptomyces Project.

** Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain AM-6424.

	Response*
Melanin formation	+**
Tyrosinase reaction	−
Nitrate reduction	−
Hydrolysis of starch	+
Liquefaction of gelatin	− (22°C)
Peptonization of milk	+ (37°C)
Coagulation of milk	− (37°C)
Cellulolytic activity	−

* +, Active; −, not active.

** Melanin formation is active in tryptone - yeast medium, but not active in tyrosine agar, peptone - yeast extract-iron agar and glucose - peptone - gelatin (stab culture).

Table 3. Utilization of carbon sources by strain AM-6424.

Carbon source	Utilization*
D-Glucose	+
D-Fructose	+
L-Rhamnose	−
L-Arabinose	+
i-Inositol	+
Raffinose	±
D-Xylose	+
Sucrose	−
D-Mannitol	+
Melibiose	+
Cellulose	−

* +, Utilized; ±, weakly utilized; −, not utilized.

Spira type. The spores are cylindrical. The spore surface is smooth with some winkled forms. Sclerotic granules, sporangia and flagellated spores were not observed.

Chemotaxonomy

The chemical analyses of sugars in whole cells and diaminopimelic acid (A_2pm) in cell wall were carried out by the methods of LECHEVALIER and LECHEVALIER.[1] Strain AM-6424 showed presence of LL-A_2pm in the cell wall and no characteristic sugar pattern.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th edition) published by Container Corporation of America. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon source each. The cultural and physiological characteristics, and the utilization of carbon sources of strain AM-6424 are shown in Tables 1, 2 and 3, respectively.

Strain AM-6424 exhibits the following properties. Sporophore, *Spira*; spore, cylindrical; spore surface, smooth; color of vegetative mycelium, gold or brown; color of aerial mycelium, white or ivory; production of melanoid pigment, active; soluble pigment, yellow or gold; A_2pm in cell wall, LL-type.

Based on the taxonomic properties described above, strain AM-6424 is considered to belong to the genus Streptomyces and to be a strain of the white series of the PRIDHAM and TRESNER grouping[4]. Strain AM-6424 has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. AM-6424 and the accession No. FERM-P 6791.

Production and Isolation

The stock culture of strain AM-6424 was inoculated into 100 ml of a seed medium consisting of 2% glucose, 0.5% meat extract, 0.5% peptone, 0.3% dried yeast, 0.5% NaCl and 0.3% $CaCO_3$ in a 500-ml Sakaguchi flask and incubated at 27°C for 48 hours. Three hundred milliliters of a thus obtained seed culture was transferred to 30 liters of a production medium in a 50-liter jar fermentor and the fermentation was carried out at 27°C with agitation of 250 rpm and aeration of 10 liters/minute. The composition of the production medium was 2% starch, 0.5% glycerol, 0.25% soybean meal, 0.5% NH_4Cl, 0.1%

K_2HPO_4 and 0.15% $Ca_3(PO_4)_2$ (presterile pH 6.8). The antibiotic production started at 15~20 hours after inoculation then gradually increased and reached a maximum (2 μg/ml) at 39 hours.

A 42-hour culture (30 liters) was clarified with a Sharples centrifuge to obtain about 28 liters of a supernatant fluid. The fluid was adjusted to pH 7.2 with 2 N NaOH and treated with 500 ml of Amberlite IRC-50 (Na^+) in a batchwise operation. The antibiotic in the effluent was adsorbed on an activated carbon column (20×9 cm), and eluted with 40% acetone. The active fractions were combined, concentrated *in vacuo* to a small volume and lyophilized to obtain 41.6 g of crude powder. The powder was chromatographed on an Avicel column (45×6.5 cm), eluting with a mixed solvent of methanol and water (6: 1). The active fractions were combined, concentrated *in vacuo* to 100 ml and lyophilized to give 820 mg of yellowish brown powder. The powder was dissolved in 1 ml of water and applied on a Sephadex LH-20 column (90×3 cm) previously equilibrated with water. The antibiotic was eluted with water. The active fractions were combined, concentrated *in vacuo* to 10 ml and lyophilized to give a pale yellow powder (40 mg). The antibiotic was then purified by preparative thin-layer chromatography (TLC) on Avicel developing with 1-butanol - acetic acid - water (3: 1: 1). The active zone was cut out from TLC and eluted with water. The eluate was concentrated *in vacuo* to 1 ml and lyophilized to give a pale yellow powder (16.6 mg). The powder was dissolved in 0.5 ml of water and chromatographed on Sephadex LH-20 column eluting with water. The active fraction were combined, concentrated *in vacuo* to 1 ml and lyophilized to yield 5.5 mg of pure karabemycin as a white powder.

The antibiotic activity was assayed by paper disc method against *Bacillus subtilis* PCI 219 on synthetic agar plates. The antibiotic was also detected by Avicel TLC: Solvent, 1-propanol - pyridine - acetic acid - water (15: 10: 3: 6); detection, ninhydrin; Rf value, 0.47.

Table 4. Physicochemical properties of karabemycin.

Appearance		White powder, weakly basic
Melting point		280~282°C (dec.)
Optical rotation		$[\alpha]_D^{20}$ −40.3° (*c* 0.55, H_2O)
Elemental analysis (%)		
Calcd. for $C_{15}H_{24}N_4O_6 \cdot 2H_2O$		C 45.91, H 7.19, N 14.28
Found		C 46.51, H 6.90, N 14.15
Molecular formula		$C_{15}H_{24}N_4O_6$
Mass FD-MS: *m/z*		357 [(M+H)$^+$]
Color reaction	(+)	Ninhydrin, RYDON-SMITH
	(−)	*p*-Anisidine, DRAGENDORFF
UV (H_2O)		End absorption
IR, ν_{max}^{KBr} cm^{-1}		3250, 2950, 1740, 1640, 1580, 1380
pKa		9.2
Solubility	Soluble	H_2O, DMSO
	Insoluble	Chloroform, ethyl acetate, benzene, *n*-hexane
Rf values (Avicel TLC)		1-PrOH - pyridine - AcOH - H_2O (15: 10: 3: 6), Rf 0.47; 1-BuOH - AcOH - H_2O (3: 1: 1), Rf 0.42
Amino acid analysis (molar ratio)		Valine (1.0), alanine (1.0), unknown (1.0)

Physicochemical Properties

Table 4 shows the physicochemical properties of karabemycin. The molecular formula of karabemycin was proposed to be $C_{15}H_{24}N_4O_6$ (MW 356) from the elemental analysis, FD-mass, and, ^1H and ^{13}C NMR. The formula $C_{15}H_{24}N_4O_6$ is supported by the FD-mass analysis of DNP-karabemycin: M$^+$ *m/z* 523 and (M+Na)$^+$ *m/z* 546. In the IR spectrum of karabemycin (Fig. 1), the presence of amide bonds was observed at 1640 cm^{-1} and 1580 cm^{-1}. The acid hydrolysate (6 N HCl, 110°C, 12 hours) gave one molar each of valine, alanine and an unknown amino acid. Figs. 2 and 3 show the ^1H and ^{13}C NMR spectra of karabemycin, respectively. The four carbon signals of amide carbonyl and ester carbon groups at 178.3, 174.5, 171.5 and 169.6 ppm were observed (Fig. 3). From the analyses mentioned above, karabemycin is considered to be a tripeptide antibiotic.

Fig. 1. IR spectrum of karabemycin (KBr).

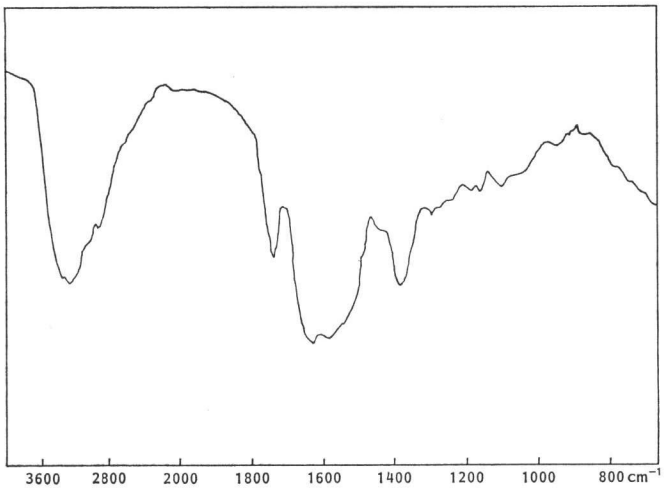


Fig. 2. ^{1}H NMR spectrum (400 MHz, D_2O) of karabemycin.

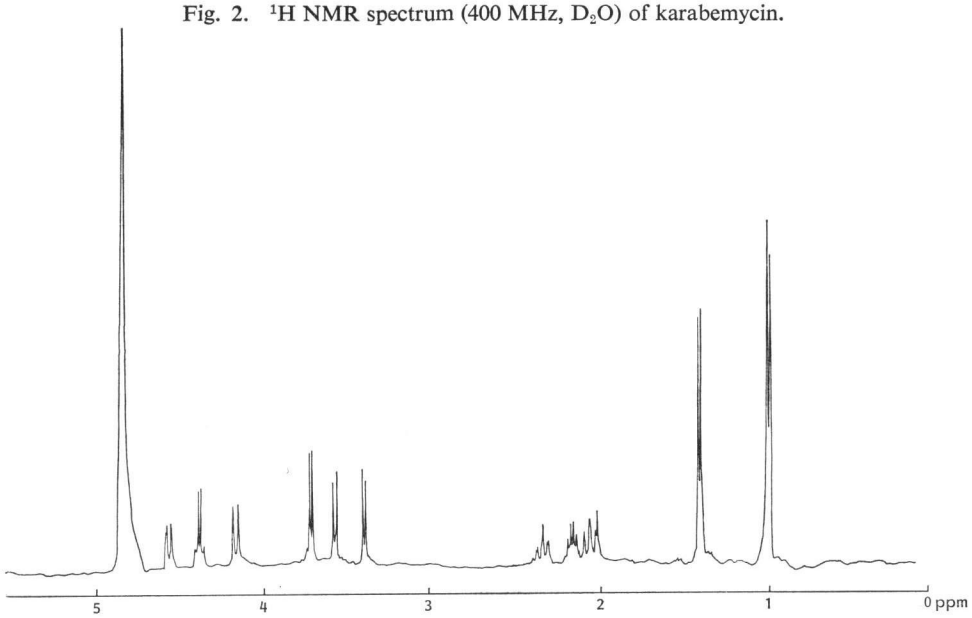


Fig. 3. ^{13}C NMR spectrum (25 MHz, D_2O) of karabemycin.

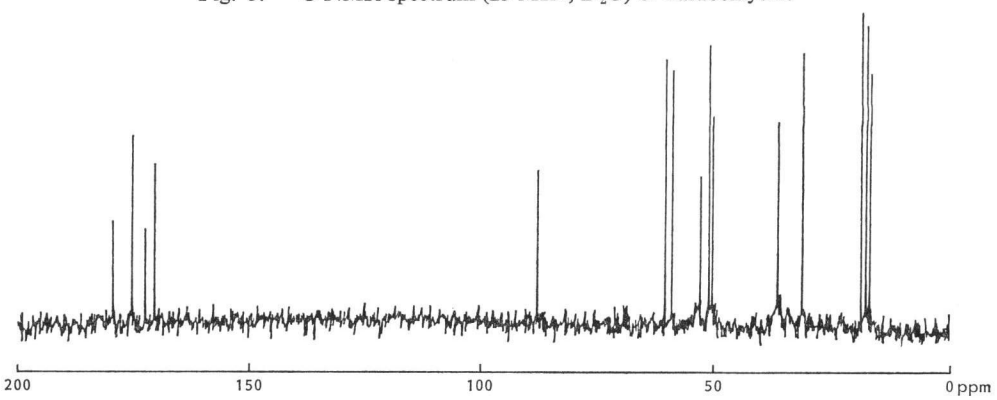


Table 5. Antimicrobial spectrum of karabemycin by paper disc method.*

Test organism	Medium**	Inhibition zone (mm)
Bacillus subtilis PCI 219	1	—
	2	40.2
Staphylococcus aureus IFO 12732	1	—
Escherichia coli IFO 12734	1	—
	2	35.4
Pseudomonas aeruginosa IFO 12689	1	15.0
	2	45.2
P. lachrymans	1	18.8
Erwinia carotovora	1	—
	2	62.4
Klebsiella pneumoniae IFO 3512	1	—
Alkaligenes faecalis	1	—
Candida albicans IFO 1060	3	—
	4	—
Aspergillus niger ATCC 6275	3	—
	5	—

* Paper disc, 8 mm in diameter; karabemycin, 1,000 μg/ml.

** 1: Nutrient agar.
2: 4 g glucose, 1 g NH_4Cl, 5.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 0.011 g $CaCl_2 \cdot 2H_2O$, 0.246 g $MgSO_4 \cdot 7H_2O$, 15 g agar, water 1,000 ml.
3: Potato - sucrose agar.
4: 20 g glucose, 2.0 g asparagine, 2.0 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.33 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 mg KI, 5 mg yeast extract, 15 g agar.
5: Czapek agar.

Table 6. Effect of karabemycin on the growth of plant roots.

Karabemycin (μg/ml)	Inhibition* (%)	
	Oryza sativa	*Raphanus sativus*
500	72.2	80.0
250	52.2	67.4
125	23.4	61.1
62.5	10.3	15.4
31.3	2.3	4.3

* Inhibition (%) = A–B/A × 100
A, root length in non-treated class
B, root length in treated class with karabemycin

Five milliliters of karabemycin aqueous solution was added to a Petri dish (7.5 cm in diameter). Five seeds of *Oryzae sativa* or *Raphanus sativus* per dish were inoculated into the Petri dish. The seeds were incubated at 25°C for 5 days under a fluorescent lamp.

Biological Properties

Table 5 shows the antimicrobial spectrum of karabemycin. The antibiotic was active against some bacteria on a synthetic agar medium, but was inactive or weakly active against bacteria on a complex agar medium and was inactive against fungi. The inhibitory activity of karabemycin against bacteria on a synthetic agar medium was reversed by L-glutamine and L-glutamic acid.

Karabemycin has inhibitory activity on the growth of plant roots (Table 6). Karabemycin (500 μg/ml) did not show any toxic symptoms to RK-13 cells grown in tissue culture.

Discussion

Karabemycin was isolated from the culture filtrate of *Streptomyces* sp. AM-6424. The antibiotic was found to be an antimetabolite of glutamine (glutamic acid), and a tripeptide antibiotic consisting of alanine, valine and an unidentified amino acid. Karabemycin was compared with known antibiotics of actinomycetes origin, such as antimetabolites of glutamine (glutamic acid) and peptide antibiotics. However, because none of their physicochemical properties was identical with those of karabemycin, it is reasonable to conclude that karabemycin is new.

A paper describing structure elucidation of karabemycin is now in preparation.

Acknowledgment

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