TAKAOKAMYCIN, A NEW PEPTIDE ANTIBIOTIC PRODUCED BY *STREPTOMYCES* SP.

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(Received for publication March 28, 1984)

A new peptide antibiotic named takaokamycin was isolated from a fermentation broth of *Streptomyces* sp. AC-1978, a soil isolate. It exhibits antibacterial activity against some Gram-positive bacteria. The molecular weight was found to be 1,130 on the basis of elemental analysis, FD-mass spectrum and ^{1}H and ^{13}C NMR. Acid hydrolysate of takaokamycin contains isoleucine, threonine and unidentified amino acids.

In the course of screening for new antibiotics of actinomycete origin, we found that a new compound active against some Gram-positive bacteria was produced by a streptomycete strain AC-1978 isolated from a soil sample collected at Takaoka City, Toyama Prefecture, Japan.

The present paper deals with the taxonomy of the strain AC-1978 and the production, isolation, and some biological and physico-chemical properties of takaokamycin.

Taxonomy of the Producing Organism

Morphology

The vegetative mycelium grows well on both synthetic and complex agar media, and does not show fragmentation into coccoid or bacillary elements. The aerial mycelium, which is velvety, is abundantly produced on inorganic salts - starch agar, tyrosine agar and nutrient agar. The spore chains are of the Spira type and have more than ten spores per chain. The spores are ellipsoidal in shape, 0.9×1.1 μm in size and have a smooth surface (Plate 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Analysis

The chemical analyses of sugars in whole cells and amino acids in cell walls were carried out by the method of LECHEVALIER and LECHEVALIER.[1]

The sugar pattern of the strain is not characteristic and LL-diaminopimelic acid (A_2pm) was detected.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and the media recommended by WAKSMAN[3] were used for these experiments. Cultures were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated are those of the Color

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Table 1. Cultural characteristics of strain AC-1978.

Medium	Cultural characteristic
Yeast extract - malt extract agar (ISP)*	G: Good, bamboo (2gc) R: Mustard tan (21g) AM: Moderate, velvety, silver gray (3fe), the edge is dark brown (3nl) SP: None
Oat meal agar (ISP)*	G: Good, penetrant, pearl pink (3ca) R: Beige brown (3ig) AM: Moderate, velvety, hygroscopic, dark brown (3nl), the edge is silver gray (3fe) SP: None
Inorganic salts - starch agar (ISP)*	G: Good, penetrant, pearl pink (3ca) R: Dark covert gray (2ih) AM: Abundant, velvety, hygroscopic, oxford gray (1) SP: None
Glycerol - asparagine agar (ISP)*	G: Good, pearl pink (3ca) R: Light wheat (2ea) AM: Moderate, velvety, white SP: None
Glucose - asparagine agar	G: Good, light ivory (2ca) R: Melon yellow (3ga) AM: Moderate, velvety, beige (3gc), the edge is white SP: None
Peptone - yeast extract - iron agar (ISP)*	G: Good, bisque (3ec) R: Cork tan (4ie) AM: Poor, velvety, white SP: None
Tyrosine agar (ISP)*	G: Good, penetrant, pearl pink (3ca) R: Beaver (3li) AM: Abundant, velvety, white and gray SP: None
Sucrose - nitrate agar**	G: Good, penetrant, pearl pink (3ca) R: Dark brown AM: Moderate, hygroscopic, dark brown (3nl) SP: None
Glucose - nitrate agar**	G: Good, penetrant, pearl pink (3ca) R: Beige brown (3ig) AM: Moderate, hygroscopic beaver (3li) SP: None
Glycerol - calcium malate agar**	G: Good, cork tan (4ie) R: Cork tan (4ie) AM: Very poor, white SP: None
Glucose - peptone agar**	G: Good, wrinkled, pearl pink (3ca) R: Light wheat (2ea) AM: None SP: None
Nutrient agar**	G: Good, penetrant, pearl pink (3ca) R: Dark brown (3nl) AM: Abundant, velvety, slightly hygroscopic, silver gray (3fe) SP: None

* Medium recommended by International Streptomyces Project.
** Medium recommended by S. A. WAKSMAN.
Abbreviation: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP; soluble pigment.

Plate 1. Scanning electron micrograph of aerial mycelia of *Streptomyces* sp. AC-1978.
Bar represents 1 μm.

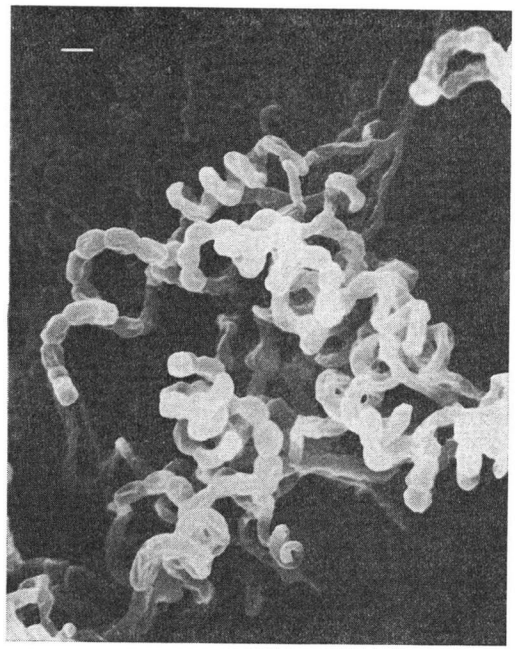


Table 2. Physiological properties of strain AC-1978.

	Response*
Melanin formation	−
Tyrosinase reaction	−
Nitrate reduction	+
Liquefaction of gelatin	+ (22°C)
Hydrolysis of starch	+
Coagulation of milk	± (37°C)
Peptonization of milk	+ (37°C)
H_2S formation	−
Cellulolytic activity	−
Temperature range for growth	15~47°C

* +, Active; −, not active.

Table 3. Utilization of carbon sources by strain AC-1978.

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

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

Harmony Manual (4th edition) published by the Container Cooperation of America. The utilization of carbon sources was tested by growth on Pridham & Gottlieb medium containing 1% carbon source each. The cultural and physiological characteristics, and the utilization of carbon sources of strain AC-1978 are shown in Tables 1, 2 and 3, respectively.

Strain AC-1978 exhibits the following properties. Sporophore, Spira; spore, ellipsoidal and smooth surface; color of aerial mycelium, white or gray to brownish; melanoid pigment, none; soluble pigment, none; A_2pm in cell wall, LL-type. Based on the taxonomic properties described above, strain AC-1978 is considered to belong to the genus *Streptomyces* being a strain of the white or gray series of the PRIDHAM and TRESNER grouping.[4]

Production and Isolation

The stock culture of strain AC-1978 was inoculated into 100 ml of a seed medium consisting of glucose 1%, dextrin 1%, NZ-amine (type A) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.1% in a 500-ml Erlenmeyer flask and incubated at 27°C for 48 hours. Six hundred milliliters of the seed culture was transferred to 20 liters of a production medium consisting of glycerol 2%, soy bean meal 0.5%, corn steep liquor 1.5%, $CaCO_3$ 0.3% and $CoCl_2 \cdot 6H_2O$ 0.0002%, pH 6.5 in a 30-liter jar fermentor and the aerobic fermentation was carried out at 30°C. The antibiotic production started at 15~20 hours after the inoculation, then gradually increased and reached maximum (27 μg/ml) at 72 hours.

The cultured broth (20 liters) was centrifuged to obtain about 19 liters of a supernatant fluid and 1 kg of wet mycelial cake. The mycelial cake was extracted with 60% aqueous acetone (5 liters). After

the removal of acetone under reduced pressure, the aqueous solution (500 ml) was extracted five times with ethyl acetate (200 ml). The supernatant fluid was also extracted two times with ethyl acetate(5 liters). The extracts were combined, concentrated *in vacuo* and then treated with *n*-hexane (200 ml) and the resulting viscous precipitate was washed with *n*-hexane to give brown paste (1.7 g). The crude material was applied to a silica gel column (40×3 cm) and eluted with $CHCl_3$ - MeOH (50: 1). The active fractions were collected and evaporated to yield 249 mg of brown powder. The powder was further chromatographed on a silica gel column with $CHCl_3$ - Me_2CO (15: 1) to give 120 mg of the pure material as white powder.

The antimicrobial activity during isolation was assayed by the paper disk method using *Micrococcus luteus* PCI 1001 as a test organism. The antibiotic was also monitored by silica gel TLC using $CHCl_3$ - MeOH (10: 1) as a developing solvent and the spot was detected at Rf 0.63 under UV light.

Physico-chemical Properties

Table 4 shows the physico-chemical properties of takaokamycin. The molecular ion peak was observed at *m/z* 1,129 in FD mass spectrum. The molecular formula was estimated as $C_{52\sim54}H_{80\sim78}N_{8\sim11}O_{16\sim19}$ containing no sulfur atom by elemental analysis, FD-MS and ^1H and ^{13}C NMR data. The molecular weight of takaokamycin was proposed to be 1,130. The IR spectrum (Fig. 1) indicated the presence of amide bond (1620 and 1549 cm^{-1}). The acid hydrolysate (6 N HCl, 105°C, 22 hours) contained isoleucine, threonine and three unidentified amino acids detected by the amino acid analyzer. Figs. 2 and 3 show the ^1H and ^{13}C NMR spectra, respectively.

Biological Properties

The antimicrobial activity of takaokamycin was determined by the conventional agar dilution method using Difco heart infusion agar. The antibiotic was active against two Gram-positive bacteria, as shown in Table 5.

The antibiotic did not show any toxic effect on mice at 500 mg/kg by ip injection.

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

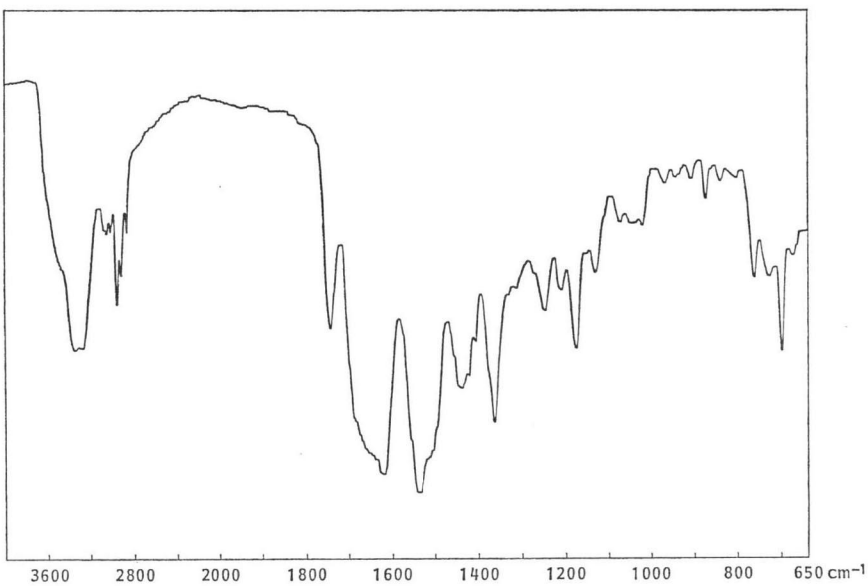


Fig. 2. ^{1}H NMR spectrum (400 MHz) of takaokamycin.

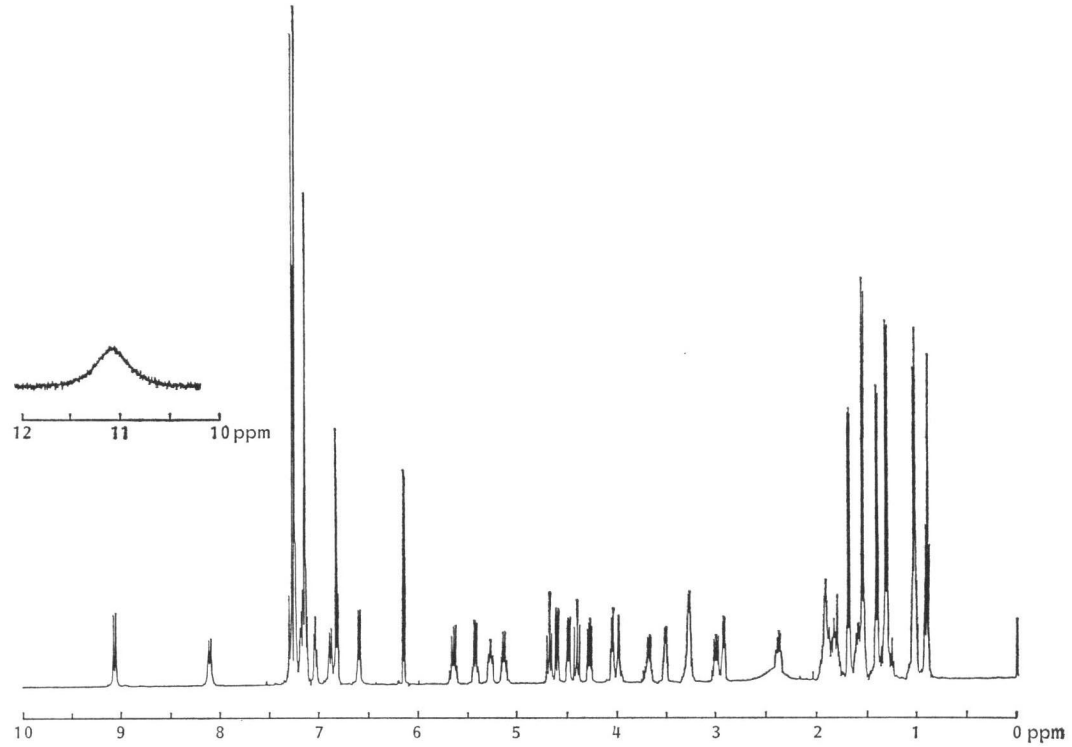


Table 4. Physico-chemical properties of takaokamycin.

Appearance	White powder
Mp	166~168°C
$[\alpha]_D^{26}$	+21.6° (*c* 1.0, MeOH)
Anal Found:	C 57.13, H 6.06, N 11.72
FD-MS *m/z*	1,129 (M), 1,152 (M+Na)
UV λ_{max} nm(ε)	279 (16,950) in MeOH 273 (18,419) in 0.01 N HCl - MeOH 281 (18,080) in 0.01 N NaOH - MeOH
IR ν_{max}^{KBr} cm^{-1}	3300, 2990, 1748, 1620, 1549, 1450, 1360
Rf values (silica gel TLC)	$CHCl_3$ - MeOH (10: 1), 0.63 Benzene - Me_2CO (3: 1), 0.44
Amino acid analysis	Isoleucine, threonine, three unidentified amino acids

Table 5. Antibacterial spectrum of takaokamycin.

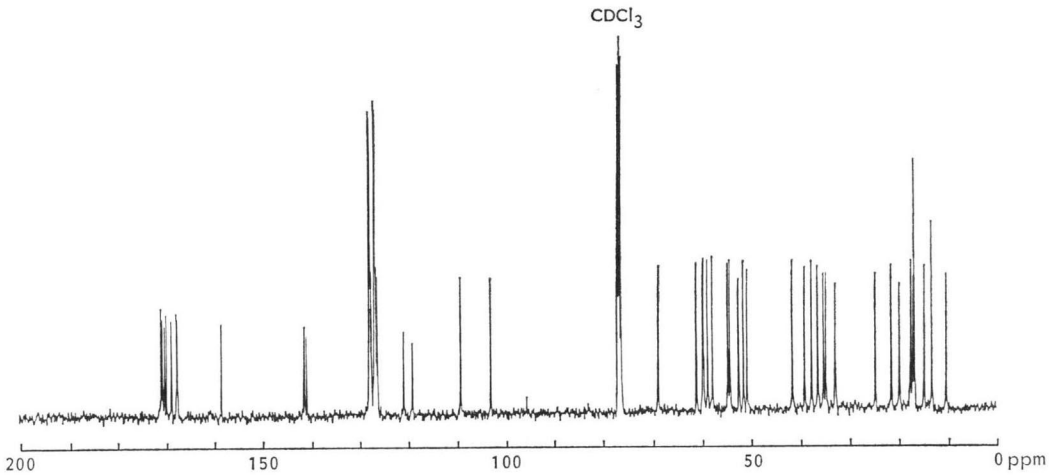
Test organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	>100
S. aureus FDA 209P	>100
Bacillus subtilis ATCC 6633	>100
B. cereus IFO 3001	12.5
Micrococcus luteus ATCC 9341	1.56
Mycobacterium smegmatis ATCC 607	>100
Escherichia coli NIHJ	>100
E. coli NIHJ JC-2	>100
Klebsiella pneumoniae ATCC 10031	>100
Proteus vulgaris IFO 3167	>100
Pseudomonas aeruginosa IFO 3080	>100

* Minimal inhibitory concentrations were assayed by agar dilution method using heart infusion agar (pH 7.0, 37°C, 20 hours).

Discussion

Takaokamycin was found to be a peptide compound consisting of isoleucine, threonine and three unidentified amino acids, and shows specific activity against *M. luteus* and low toxicity. Among known antibiotics, there are no compounds having physico-chemical and biological properties identical with those of takaokamycin. Consequently, the antibiotic was concluded to be a new compound. The structure elucidation is now in progress.

Fig. 3. ^{13}C NMR spectrum (100.6 MHz) of takaokamycin.



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