FL-120A~D', NEW PRODUCTS RELATED TO KINAMYCIN FROM *Streptomyces chattanoogensis* subsp. *taitungensis* subsp. nov.

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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Six new kinamycin antibiotics, designated as FL-120A~D' (**1**~**6**) were isolated from the culture filtrate of *Streptomyces* sp. strain IY2-13. Based on its cultural, physiological, morphological and chemical characteristics, this strain was identified as a new subspecies of *Streptomyces chattanoogensis* and named *S. chattanoogensis* subsp. *taitungensis*. These kinamycins have demonstrated a potent activity against Gram-positive aerobic and anaerobic bacteria.

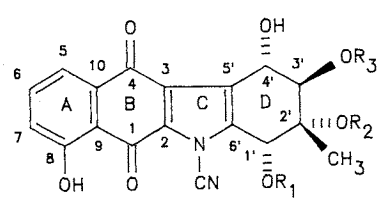
The kinamycins are a group of antibiotics having a novel benz[*b*]-tetrahydrocarbazole skeleton and *N*-cyano moiety. They were first isolated and characterized by ŌMURA *et al.*[1~4] in 1970, and were shown to be active against Gram-positive bacteria, but less so against Gram-negative organisms. In the course of our screening program for new bioactive substances, six new members (**1**~**6**, Fig. 1) of the kinamycin family have been isolated from the culture broth of *Streptomyces* sp. strain IY2-13. These structures represent the first reported epoxide kinamycin (**2**, **3**) and new propionyl derivative of kinamycin (**5**), and new isobutyryl derivatives of kinamycin (**1**, **4**, **6**). This paper describes the taxonomy of the producing strain, fermentation, and antimicrobial activity of these compounds. The isolation and structure determination will be discussed in the following paper[5].

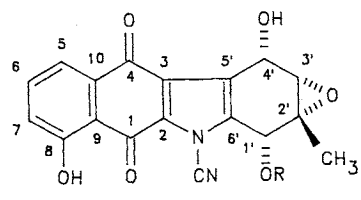
Materials and Methods

Bacterial Strains

Strain IY2-13 was isolated from a Sea-wall soil sample collected in Taitung County, Taiwan Province,

Fig. 1. Structures of FL-120A~D' (**1**~**6**) and kinamycin D (**7**).





1 R_1=Ac, R_2=$COCH(CH_3)_2$, R_3=Ac
4 R_1=$COCH(CH_3)_2$, R_2=H, R_3=Ac
5 R_1=$COCH_2CH_3$, R_2=H, R_3=Ac
6 R_1=$COCH(CH_3)_2$, R_2=H, R_3=H
7 R_1=R_3=Ac, R_2=H

2 R=$COCH(CH_3)_2$
3 R=Ac

R.O.C. Strain IY2-13 has been deposited in Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, R.O.C., the accession number is CCRC 15124. *Streptomyces chattanoogensis* ATCC 13358^T, and the bacterial strains used for determination of the antibacterial spectrum of kinamycins were from the American Type Culture Collection (ATCC).

Taxonomic Studies

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[6]. The media were purchased from Difco laboratories. For the evaluation of cultural characteristics, the strains were incubated for 14~21 days at 27°C. Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[7]. The analysis of cell wall and whole cell composition followed the methods of BECKER *et al.*[8]. The electrophoretic protein patterns of the strains was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the methods of THOMPSON *et al.*[9].

Media and Fermentation

To prepare vegetative inoculum for antibiotic production, the strain was grown in a seed medium with the following composition: dextrose (Difco) 2%, Pharmamedia (Sigma) 0.5%, yeast extract (Difco) 0.5%, $MgSO_4 \cdot 7H_2O$ (Wako) 0.01%, K_2HPO_4 (Wako) 0.01%. The pH of the medium was adjusted to 7.1 before sterilization. After 24 hours of growth at 27°C on an orbital shaker (diameter 70 mm), the culture was taken as inoculum seed to inoculate into a Chemap-CF 150 fermentor containing 100 liters of production medium with the following compositions: soybean meal (Sigma) 1.5%, glycerol (Wako) 0.25%, dextrose (Difco) 1.5%, NaCl (Merck) 0.5%, corn meal (Sigma) 0.5%, $CaCO_3$ (Merck) 0.01% in tap water. The medium was adjusted to pH 7.1 before sterilization. The fermentation was carried out at 27°C for 48 hours (aeration: 30 liter/minute, agitation: 200 rpm). The antibiotic production was monitored by a paper-disc assay using *Staphylococcus aureus* ATCC 6538p as test organism.

HPLC Analysis

FL-120A, B, B', C, C', D and D' were individually identified by analytical HPLC on a Beckman 165 μ-porasil (No. 100) with guard column, using a mobile phase of chloroform-ethylacetate 95:5 in the first 12 minutes, then changed to 65:35 in the following 4 minutes. The flow rate was controlled at 0.6 ml/minute, and the effluent was monitored at 254 nm.

Antibacterial Activity

The antibacterial spectra of purified compounds were determined by a conventional dilution method with Mueller-Hinton broth (Difco) or the medium suitable for the test organism growth. Minimum inhibition concentration (MIC) was expressed in terms of μg/ml after overnight incubation at 37°C. Due to poor solubility in water, the purified compounds were initially dissolved in trace amount of dimethyl sulfoxide (DMSO, Merck) and subsequently diluted with medium.

Results and Discussion

Taxonomic Studies of the Producing Strain

Morphological observations were made with both light and electron microscopes of various cultures grown at 27°C for 14~21 days, the mature spores of strain IY2-13 occurred only on the ISP 4 agar plate. The spore chains consisted of more than 10 spores forming tight spirals (Fig. 2). The spores were oval and $0.5 \sim 0.7 \times 1.0 \sim 1.4$ μm in size and had a spiny surface. Sclerotic granule, sporangia and flagellated spores were not observed.

The vegetative mycelia grew abundantly on ISP medium 2 and ISP medium 4, moderately on ISP medium 3, ISP medium 5, and ISP medium 7, poorly on ISP medium 6 and Czapek solution agar (Table 1).

From the aerial mycelium mass color produced on ISP medium 4, this strain could be classified in

the gray series.

The cell wall analysis of strain IY2-13 showed that is contained LL-diaminopimelic acid and glycine. Accordingly, the cell wall of this strain belongs to type I[10].

Physiological properties of the strain were shown in Table 2. Gelatin liquefaction, milk peptonization and starch hydrolysis were positive.

The results of utilization of carbon sources are summarized in Table 2.

Microscopic studies and cell wall analysis of strain IY2-13 indicated that the strain was classified in the genus *Streptomyces*. Therefore, the strain was compared with the published description of various

Fig. 2. Scanning electron micrograph of a spore chain of strain IY2-13.

Bar represents 2.0 μm.

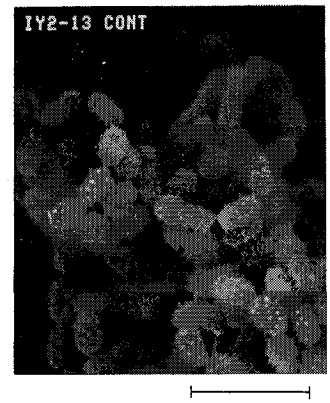


Table 1. Cultural characteristics of strain IY2-13 and *Streptomyces chattanoogensis* ATCC 13358^T on various media.

		IY2-13	*S. chattanoogensis*
Yeast extract-malt extract agar (ISP-2)	G:	Abundant, yellowish brown	Abundant, brown
	R:	Yellowish brown	Brown
	AM:	None	Few white spots
	SP:	Yellowish brown	Brown
Oatmeal agar (ISP-3)	G:	Moderate, pale yellowish brown	Moderate, brown
	R:	Pale yellowish brown	Brown
	AM:	None	None
	SP:	Pale yellowish brown	Brown
Inorganic salts-starch agar (ISP-4)	G:	Abundant, brown	Abundant, grayish yellow
	R:	Brown	Grayish yellow
	AM:	Moderate, grayish white	Moderate, grayish yellow
	SP:	Brownish	Light yellow
Glycerol-asparagine agar (ISP-5)	G:	Moderate, brown	Moderate, yellowish brown
	R:	Brown	Yellowish brown
	AM:	None	Few white spots
	SP:	None	Light yellowish green
Peptone-yeast extract-iron agar (ISP-6)	G:	Poor	Poor
	R:	Indistinct	Indistinct
	AM:	None	None
	SP:	None	None
Tyrosine agar (ISP-7)	G:	Moderate	Moderate
	R:	Pale brownish	Pale brownish
	AM:	None	None
	SP:	Pale brownish	Pale brownish
Czapek solution agar	G:	Poor	Excellent
	R:	Indistinct	Indistinct
	AM:	None	Few white spots
	SP:	None	None

G: Growth of vegetative mycelium.
R: Reverse color of the culture plate.
AM: Aerial mycelium.
SP: Soluble pigment.

Table 2. Comparison of IY-2-13 with *Streptomyces chattanoogensis* ATCC 13358^T.

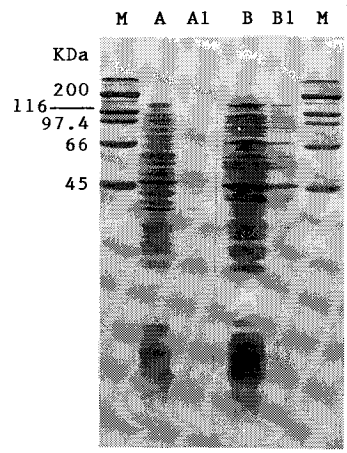
Property	IY2-13	*S. chattanoogensis*	Property	IY2-13	*S. chattanoogensis*
Spore chain	Spirals	Spirals	Utilization of carbon source:		
Spore surface	Spiny	Spiny	D-Glucose	+	+
Aerial mycelium	Grayish white	Grayish white	D-Xylose	−	−
Vegetative mycelium	Yellowish brown	Yellowish brown	L-Arabinose	−	−
Soluble pigment	Brown	Yellowish green	L-Rhamnose	−	−
Melanin formation	−	−	D-Fructose	+	+
Starch hydrolysis	+	+	D-Galactose	+	+
Peptonization of milk	+	−	Raffinose	−	+
			D-Mannitol	+	+
Liquefaction of gelatin	+	−	Inositol	+	+
			Salicin	−	−
NaCl tolerance	<7%	7~10%	Sucrose	−	+
Streptomycin tolerance[a]	−	−	Antibiotic produced	Kinamycins	Pimaricin[16]
Growth on Czapek solution agar	Poor	Excellent			

[a] Streptomycin: 100 μg/ml.
+: Active.
−: Not active

Streptomyces species, and the results showed that strain IY2-13 was considered to most resemble to *Streptomyces chattanoogensis* ATCC 13358^T[12~15].

The taxonomic characteristics of strain IY2-13 were compared directly with those of *S. chattanoogensis* ATCC 13358^T in detail. As shown in Tables 1 and 2, both strains almost have the same cultural characteristics, but there are some differences in physiological properties and carbohydrate utilization. Tests for peptonization of milk and liquefaction of gelatin are positive for the strain IY2-13; those are negative for ATCC 13358^T. Strain IY2-13 is unable to utilize raffinose and sucrose; ATCC 13358^T is able to utilize those carbohydrates. Strain IY2-13 grows poorly on Czapek solution agar; ATCC 13358^T grows excellently. On NaCl tolerance test, strain IY2-13 is less 7%; ATCC 13358^T is between 7% and 10%. Strain IY2-13 produces kinamycins; ATCC 13358^T dose not. Comparison of the electrophoretic protein patterns shown in Fig. 3, strain IY2-13 and ATCC 13358^T have many protein bands in common, as proposed by GOTTLIEB[11], it can serve as an aid to confirm the close relationship between those two strains. From the results described above, strain IY2-13 is considered as a new subspecies of *S. chattanoogensis*. Since strain IY2-13 was isolated from the soil sample of Taitung County, Taiwan,

Fig. 3. Protein electrophoregrams of strain IY2-13 and *S. chattanoogensis*.



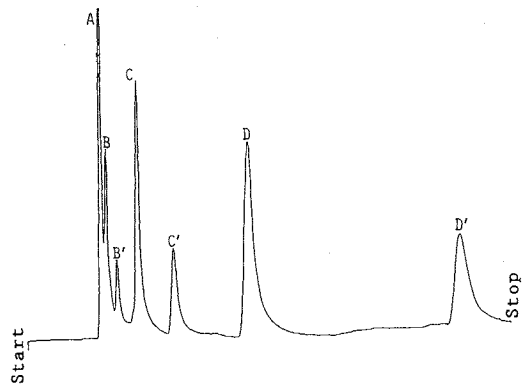
M: Molecular weight standards
A: Crude extract of strain IY2-13
AI: 1:5 dilution of A
B: Crude extract of *S. chattanoogensis* ATCC 13358^T
B1: 1:5 dilution of B

R.O.C., therefore, the name of *S. chattanoogensis* subsp. *taitungensis* subsp. nov. is proposed. The strain IY2-13 has been deposited in the Culture Collection And Research Center, Food Industry Research And Development Institute, Hsinchu, Taiwan, R.O.C. with the accession number CCRC 15124.

Production and HPLC Analysis

Strain IY2-13 was cultured in a 150 liters Chemap CF-150 fermentor at 27°C for 48 hours as described. Antibiotic production started at 20~24 hours after inoculation, then gradually increased and reached a maximum after 48~50 hours. From the culture filtrate, designated as FL-120, seven kinamycin antibiotics were isolated and purified as FL-120A, B, B', C, C', D and D' sequentially. The isolation procedures will be described in the following paper[5]. These compounds were individually identified by HPLC analysis (Fig. 4), and submitted to instrumental analysis. After comparison with the published data[1~4], FL-120D (**7**) was identified with kinamycin D, the others (**1~6**) were found to be new kinamycin antibiotics.

Fig. 4. Analytical HPLC profile of FL-120A~D'.



Name	Rt (minutes)
FL-120A (**1**)	5.63
FL-120B (**2**)	6.08
FL-120B' (**3**)	6.88
FL-120C (**4**)	8.47
FL-120C' (**5**)	11.23
FL-120D (**7**)	16.93
FL-120D' (**6**)	33.1

Biological Properties

The antimicrobial spectra of FL-120A~D' (**1~6**) and kinamycin D (**7**) are shown in Table 3. These antibiotics exhibited potent antibacterial

Table 3. Antimicrobial spectra of FL-120A~D' (**1~6**) and kinamycin D (**7**).

Organism	MIC (μg/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Staphylococcus aureus ATCC 6538p	0.05	0.4	0.01	0.01	0.01	0.03	0.03
Bacillus cereus ATCC 11778	0.25	1.6	0.05	0.03	0.03	0.1	0.02
Escherichia coli ATCC 25922	>50	>50	50	>50	50	12.8	6.4
Salmonella typhi ATCC 6539	>50	>50	50	>50	>50	6.4	6.4
Pseudomonas aeruginosa ATCC 27853	>50	>50	>50	>50	>50	>50	>50
Streptococcus pneumoniae ATCC 6303	0.39	0.78	ND	0.78	ND	ND	0.1
Bacillus anthracis ATCC 14578	0.01	0.01	ND	0.02	ND	ND	0.05
Corynebacterium diphtheriae ATCC 27011	0.2	0.2	ND	0.1	ND	ND	0.1
Listeria monocytogenes ATCC 15313	0.78	0.39	ND	0.1	ND	ND	0.1
Brucella abortus ATCC 11192	0.39	0.2	ND	1.56	ND	ND	0.78
Francisella tularensis ATCC 29684	0.78	0.39	ND	0.39	ND	ND	0.78
Haemophilus influenzae ATCC 19418	>50	25	ND	6.25	ND	ND	6.25
Klebsiella pneumoniae ATCC 13883	>50	>50	ND	>50	ND	ND	3.13
Vibrio cholerae ATCC 14035	>50	>50	ND	1.56	ND	ND	0.78
Clostridium tetani ATCC 10779	0.1	0.2	ND	0.39	ND	ND	0.2
Neisseria gonorrhoeae ATCC 23050	0.1	0.02	ND	0.13	ND	ND	0.02

ND: Not determined.

activity against Gram-positive aerobic and anaerobic organisms with MIC values less than 0.25 μg/ml, except FL-120B (**2**). No activity was observed against Gram-negative organisms. Among these seven compounds, **3**, **4**, **5** and **7** are more active than the others. The acute toxicity (LD_{50}) in mouse of FL-120A (**1**) is 35.36 mg/kg, FL-120B (**2**) > 50 mg/kg, and FL-120C' (**5**) is 6.97 mg/kg intravenously.

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