FR109615†, A NEW ANTIFUNGAL ANTIBIOTIC FROM *STREPTOMYCES SETONII*

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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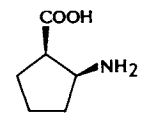
FR109615, a new antibiotic active against *Candida*, was isolated from *Streptomyces setonii* No. 7562. Based on the spectroscopic data, the structure of FR109615 was elucidated as *cis*-2-aminocyclopentane-1-carboxylic acid (**1**). The compound showed the excellent *in vivo* efficacy in a generalized infection test of mice.

In the course of our screening for new antibiotics, we found that a strain of *Streptomyces* produces an antifungal antibiotic. Although this compound **1** had been chemically synthesized before[1,2], it is for the first time that it was isolated as a microbial product and revealed to have anti-*Candida* activity. This paper describes the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and antifungal activity of FR109615.

Fig. 1. The structure of FR109615.



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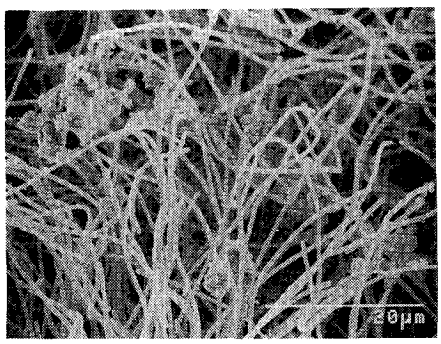
Taxonomy

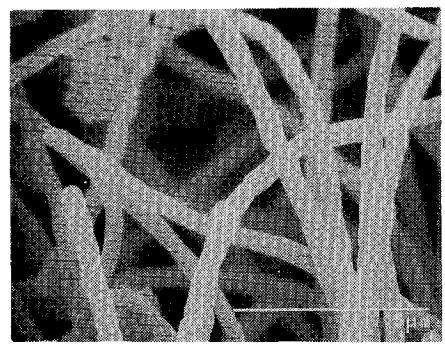
Strain No. 7562 was isolated from a soil sample

Fig. 2. Scanning electron microphotography of strain No. 7562 grown on yeast extract-malt extract agar at 30°C for 14 days.

(A) Aerial mycelia



(B) Spore chains



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obtained at Imaichi city, Tochigi Prefecture, Japan. The methods described by SHIRLING and GOTTLIEB[3] were employed for the taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 21 days on yeast extract - malt extract agar, glucose - asparagine agar and BENNET agar. Branching type of sporophores was monopodial and the form of mature sporophores was *Rectiflexibiles* with 10 to 30 spores in each chain. The spores were determined by electron microscopy (Fig. 2) to be cylindrical and measured $0.4 \sim 0.6 \times 0.8 \sim 2.2\ \mu m$ in size. Spore surfaces were smooth. Neither fragmentation of hyphae nor formation of spores occurred in the substrate mycelium. Sporangia, sclerotia and zoospores were not observed.

Cultural characteristics were observed on several media described by SHIRLING and GOTTLIEB[3], and WAKSMAN[4]. Incubation was carried out at 30°C for 21 days. The color names used in this study were taken from Methuen Handbook of Colour[5]. The aerial mass color belonged to white color series when grown on yeast extract - malt extract agar and glucose - asparagine agar. Soluble pigment was not produced. Results are shown in Table 1.

Wall analysis was performed by the methods of BECKER *et al.*[6] and YAMAGUCHI[7]. Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Physiological properties of strain No. 7562 were as follows. Temperature range for growth was determined on yeast extract - malt extract agar. Summarized physiological properties of strain No. 7562 are shown in Table 2. Temperature range for growth was from 15 to 34°C with optimum temperature

Table 1. Cultural characteristics of strain No. 7562.

Yeast extract - malt extract agar (ISP medium 2)	G: Good AM: Moderate, white RS: Light yellow SP: None	Glucose - asparagine agar	G: Good AM: Moderate, white RS: Pale yellow SP: None
Oatmeal agar (ISP medium 3)	G: Poor AM: None RS: Colorless SP: None	Nutrient agar	G: Poor AM: None RS: Colorless SP: None
Inorganic salts - starch agar (ISP medium 4)	G: Poor AM: None RS: Colorless SP: None	BENNET agar	G: Good AM: Moderate, white RS: Pale yellow SP: None
Tyrosine agar (ISP medium 7)	G: Good AM: Moderate, white RS: Light yellow SP: None	Sucrose - nitrate agar	G: Poor AM: None RS: Colorless SP: None

G: Growth, AM: aerial mycelium, RS: reverse side color, SP: soluble pigment.

Table 2. Physiological characteristics of strain No. 7562.

Temperature range for growth	15 ~ 34°C
Optimum temperature for growth	25 ~ 30°C
Liquefaction of gelatin	Positive
Coagulation of milk	Negative
Peptonization of milk	Negative
Hydrolysis of starch	Positive
Melanoid production	Negative
Decomposition of cellulose	Negative

Table 3. Utilization of carbon sources by strain No. 7562.

D-Glucose	+
D-Xylose	+
Inositol	−
Mannitol	−
D-Fructose	+
L-Rhamnose	+
Sucrose	−
Raffinose	−

−: No growth, +: good growth.

from 25 to 30°C. Gelatin liquefaction was positive. Production of melanoid pigment was negative on tyrosine agar.

Utilization of carbon was examined according to the method of PRIDHAM and GOTTLIEB[8]. The results were determined after 14 days incubation at 30°C. This strain could utilize D-glucose, L-rhamnose, D-xylose and D-fructose. Results were shown in Table 3.

The microscopic studies and cell wall composition of this strain showed that it belongs to the genus *Streptomyces* Waksman and Henrici 1943, 339. Accordingly, a comparison of this strain was made with published descriptions[9~13] of various *Streptomyces* species. Strain No. 7562 was considered to resemble *Streptomyces setonii* Waksman. Therefore, strain No. 7562 was compared with *S. setonii* IFO 13085. No significant difference was observed between the two cultures and the properties of strain No. 7562 showed good agreement with those of *S. setonii*. Therefore, strain No. 7562 was identified as a strain of *S. setonii*, and was designated as *S. setonii* No. 7562. It has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession No. FERM BP-1868.

Fermentation

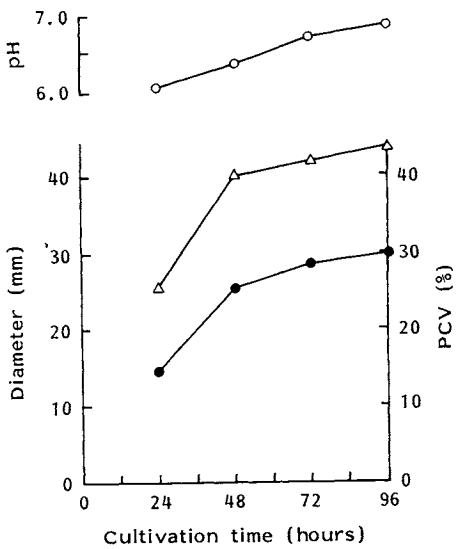
A seed medium (160 ml) consisted of corn starch 2%, cotton-seed flour 1% and dried yeast 1% was poured into each of two 500-ml Erlenmeyer flasks and sterilized. A loopful of slant culture of *S. setonii* No. 7562 was inoculated to each of the medium and cultured under shaking condition at 30°C for 2 days.

A production medium (20 liters) consisted of corn starch 2%, cotton-seed flour 0.5%, gluten meal 0.5%, corn steep liquor 0.25% and dried yeast 0.25% (pH 6.8) was poured into a 30-liter jar fermenter and sterilized.

The resultant seed culture broth (320 ml) was inoculated to the production medium and cultured at 30°C for 4 days, agitated at 200 rpm and aerated at 20 liters per minute. A typical time course for the fermentation is shown in Fig. 3. The antibiotic production started at 24 hours after inoculation, reaching a maximum at 96 hours. The amount of antibiotic produced was determined by a paper-disk agar diffusion method using *Candida albicans* as the test organism.

Fig. 3. A typical time course of fermentation by strain No. 7562.

● Potency, ○ pH, △ packed cell volume (PCV).



Isolation

The procedure for purification of FR109615 is outlined in Fig. 4. The cultured broth (20 liters) was filtered with the aid of diatomaseous earth. The filtrate (15 liters) was passed through a column of a

Fig. 4. Isolation procedure of FR109615.

Culture filtrate (15 liters)
↓
Diaion SK-1B (H^+)
↓ eluted with 0.5 N NH_4OH
Dowex 1X2 (OH^-)
↓ eluted with 0.05 N AcOH
Silica gel 60
↓ eluted with 90% aqueous 2-PrOH
Crystals (800 mg)

cation exchange resin, Diaion SK-1B (H^+ type, 600 ml). The column was washed with water and eluted with 0.5 N ammonium hydroxide. The eluate was passed through a column of an anion exchange resin, Dowex 1X2 (OH^- type, 300 ml). The column was washed with water and eluted with 0.05 N acetic acid. The eluate was concentrated under reduced pressure to dryness. The resultant crude powder (3 g) was applied on a column of silica gel 60 (150 ml) and developed with 90% aqueous 2-propanol. FR109615 was eluted in the fractions from 630 to 990 ml. The active fractions were concentrated under reduced pressure to give 1.2 g of crude crystals, then recrystallized from hot ethanol to give 0.8 g of colorless crystals.

Physico-chemical Properties

The physico-chemical properties of FR109615 are summarized in Table 4. It is soluble in water, but insoluble or sparingly soluble in methanol, acetone, ethyl acetate and chloroform. This compound gives positive color reactions for ninhydrin and $KMnO_4$, but not for Molisch and Dragendorff. The IR, ^1H and ^{13}C NMR spectra are shown in Figs. 5~7, respectively.

From these physico-chemical properties and spectroscopic data, the chemical structure of FR109615 was deduced to be **1** and confirmed by synthesis. Detailed structural study to determine an absolute configuration of **1** will be reported elsewhere.

Biological Properties

Antimicrobial activity of FR109615 was measured by the micro-broth dilution method using 96 well multi-trays. The results are shown in Table 5. Protective effect of FR109615 against systemic infection of *C. albicans* was determined as follows: ICR mice (female, 4 weeks old, 7 animals per group) were intravenously injected with 5×10^6 *C. albicans* FP 633. Therapies were administrated subcutaneously or orally 1 hour after challenge and twice a

Table 4. Physico-chemical properties of FR109615.

MP	195~196°C (dec)
Molecular formula	$C_6H_{11}NO_2$
Anal Calcd for:	C 55.79, H 8.58, N 10.85
Found:	C 56.16, H 8.29, N 10.59
MW (FAB-MS *m/z*)	130 ($M^+ + H$)
$[\alpha]_D^{20}$ (H_2O)	−9.8° (*c* 1.0)
UV $\lambda_{max}^{H_2O}$ nm (ε)	End absorption
Rf value[a] (I)	0.27
(II)	0.46

[a] Silica gel TLC (Merck Art. No. 5715), solvent (I) BuOH-AcOH-H_2O (4:1:2), (II) 2-PrOH-H_2O (75:25).

Fig. 5. IR spectrum of FR109615 (Nujol).

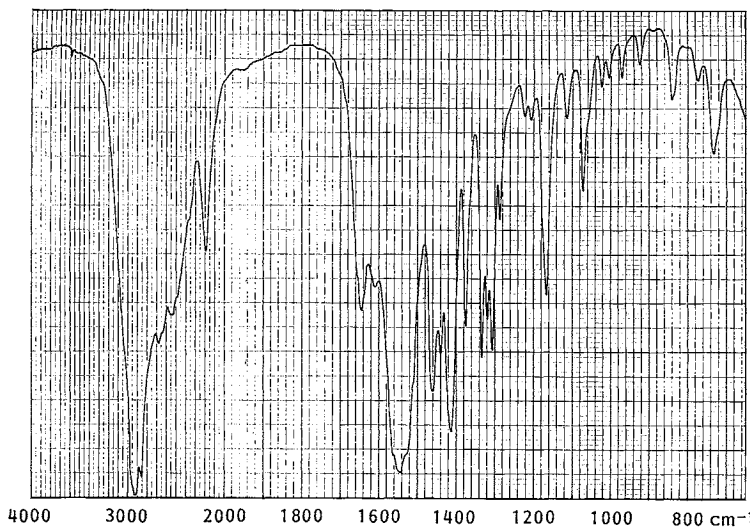


Fig. 6. ^{1}H NMR spectrum of FR109615 (400 MHz, D_2O).

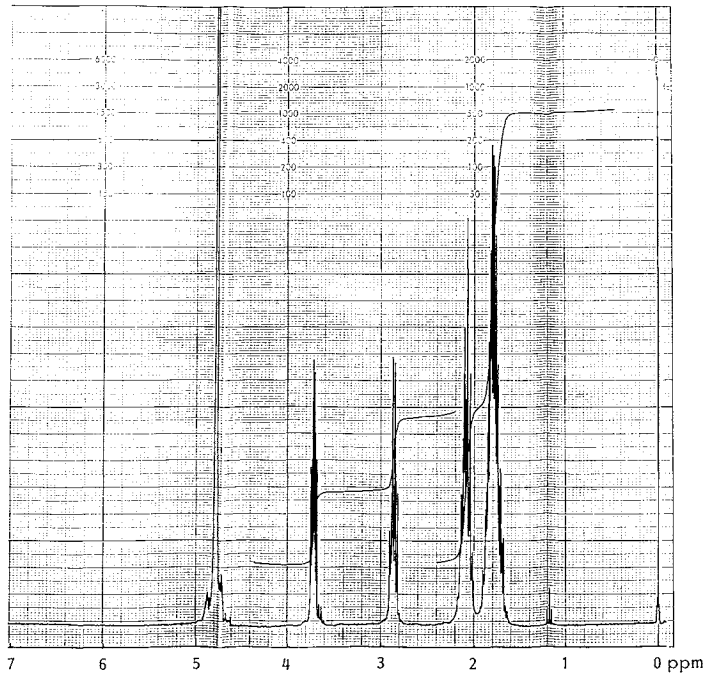


Fig. 7. ^{13}C NMR spectrum of FR109615 (67.8 MHz, D_2O).

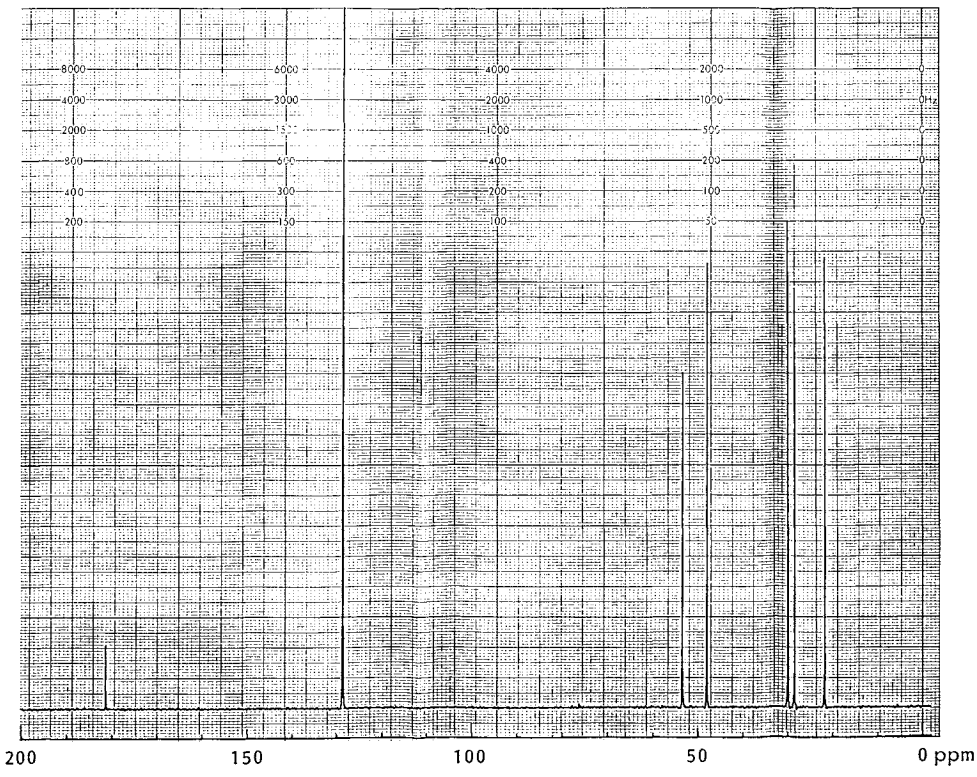


Table 5. Antimicrobial spectrum of FR109615.

Test organism	MIC (μg/ml)			Test organism	MIC (μg/ml)		
	FR109615	AMPB	KCZ		FR109615	AMPB	KCZ
Candida albicans FP 578	16	0.6	125	*C. utilis* YC 123	32	0.4	<0.5
C. albicans FP 579	32			*Mucor rouxianus* FZ 033	500	6.3	250
C. albicans FP 581	16			*Tricophyton mentagrophytes* FD 191	250	0.1	8
C. albicans FP 582	8						
C. albicans FP 629	32			*Cryptococcus albidus* YC 201	>500	1.6	>250
C. albicans FP 633	32	0.8	125				
C. krusei YC 109	8	1.6	8	*Aspergillus fumigatus* FD 050	>500	1.6	250
C. tropicalis YC 118	>500	0.4	<0.5				

The test organisms were grown in EAGLE minimum essential medium at 37°C for yeasts, at 30°C for fungi under 5% CO_2 condition.

AMPB: Amphotericin B, KCZ: ketoconazole.

Table 6. Protective effect of FR109615 against systemic infection of *Candida albicans* FP 633.

Route	Therapy (mg/kg)		Survivors/treated	Route	Therapy (mg/kg)		Survivors/treated
sc	FR109615	20	7/7		KCZ	20	3/7
		4	5/7		Vehicle		0/7
		0.8	6/7	po	FR109615	20	7/7
	AMPB	20	6/7		AMPB	20	7/7
		4	5/7		KCZ	20	0/7
		0.8	6/7		Vehicle		0/7

MIC: FR109615 32 μg/ml, amphotericin B (AMPB) 0.8 μg/ml, ketoconazole (KCZ) 125 μg/ml.

Table 7. Appearance of viable *Candida albicans* FP 633 cells in kidney from mice survived at day-9 after infection.

Group			No. of survived mice	No. of ($\times 10^{-3}$) viable cells/kidney
FR109615	20	(mg/kg, sc)	7	0, 0, 0, 0, 0, 0, 32
	4		5	12, 20, 122, 174, 274
Amphotericin B (AMPB)	20		6	0, 0, 0, 0, 0, 42
	4		5	0, 0, 18, 18, 69
Ketoconazole (KCZ)	20		3	320, 1,200, 1,500
FR109615	20	(mg/kg, po)	7	8, 9, 22, 41, 53, 88, 464
AMPB	20		7	0, 0, 0, 0, 0, 0, 0

day for 2 consecutive days. The results are shown in Table 6. *In vitro* activity of FR109615 is inferior to those of amphotericin B. Nevertheless, FR109615 has an excellent efficacy comparable to those of amphotericin B in protecting mice from *C. albicans* infection. It was confirmed by an additional experiment, that is, the viable cells of *C. albicans* in kidney from mice which survived a infection with therapies were almost extinguished (Table 7).

Thus, FR109615 showed a better *in vivo* effect than those expected from the antimicrobial activity *in vitro*. This contradiction can be partly explained by the long duration of the active form when FR109615 is administrated *in vivo*. Detailed pharmaco-kinetic study of FR109615 will be reported elsewhere.

The acute toxicity of FR109615 was determined to ICR mice (female, 4 weeks old) by a single intravenous injection. No toxic symptom was observed at the dose of 1 g/kg.

Conclusion

FR109615 has a simple and unique structure as compared with the known antifungal substances. Anti-inflammatory, anti-pyretic, analgesic, narcosis potentializing activities[14] as well as inhibitory activity on γ-aminobutyric acid uptake in synaptosomes[15,16] of **1** have been known before. Recently, it was reported[17] that chemically synthesized **1** has antimicrobial activity against a certain phathogenic fungi. But, anti-*Candida* activity is first described in this communication. The good adsorption and safety of FR109615 to animal may promise a chemotherapeutic use in human.

After preparing this paper, we noticed that Bristol-Myers group intended to report the compound[18], cispentacin, which was identical with FR109615.

Acknowledgments

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