FR112123, A NEW OLIGOPEPTIDE ANTIBIOTIC FROM *STREPTOMYCES VIRIDOCHROMOGENES*

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

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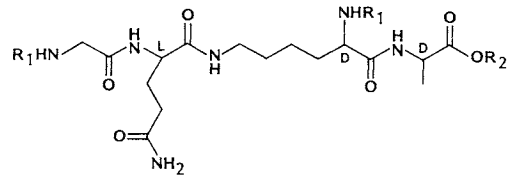
FR112123 is a new oligopeptide antibiotic produced by *Streptomyces viridochromogenes* No. 7587. The structure of FR112123 is elucidated as *N*-(N^6-(N^2-glycyl-L-glutaminyl)-D-lysyl)-D-alanine (**1**) by spectroscopic and chemical evidence. It resembles a partial structure of peptidoglycan in bacteria. The compound has a superior activity against an *Escherichia coli* mutant sensitive to inhibitors of cell wall synthesis, although it has a weak activity against the parent strain. These suggest that FR112123 might act on the biosynthesis of bacterial cell wall.

In the course of our screening for new antibiotics from *Streptomyces*, FR112123 was isolated and characterized. This paper describes the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structural elucidation and biological properties of FR112123.

Taxonomy

Strain No. 7587 was isolated from a soil sample obtained at Atami city, Shizuoka Prefecture, Japan. The methods described by SHIRLING and GOTTLIEB[1] were employed for the taxonomic study. Morphological observations were made with light and electron microscopes (Fig. 1) from cultures grown at 30°C for 21 days on yeast extract-malt extract agar, inorganic salts-starch agar, oatmeal agar and glucose-asparagine agar. The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially with sporophores forming spore chains with 20 to 60 spores per chain. The form of aerial mycelium and spore chains was *Spira*. The spores are oval ($0.5 \sim 0.7 \times 0.8 \sim 1.1\ \mu m$) with spiny surface under an electron microscope. Sporangia, flagellated spores, sclerotia and other special morphological features were not observed.

Cultural characteristics were observed on several media described by SHIRLING and



FR112123 (**1**) $R_1 = R_2 = H$
Boc-FR112123-Me (**2**) R_1 = Boc $R_2 = CH_3$

Fig. 1. Scanning electron microphotography of aerial mycelia of strain No. 7587 (Bar = 5 μm).

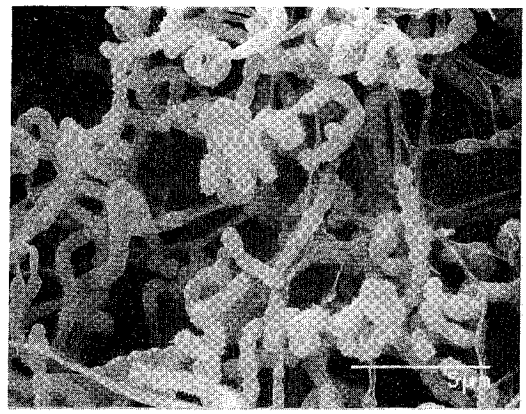


Table 1. Cultural characteristics of strain No. 7587.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar (ISP medium 2)	G: Good AM: Abundant, bluish gray RS: Brownish orange SP: Pale orange	Nutrient agar	G: Moderate AM: Thin, white RS: Light yellow SP: None
Oatmeal agar (ISP medium 3)	G: Good AM: Abundant, bluish gray RS: Grayish green SP: None	BENNET's agar	G: Moderate AM: Moderate, bluish gray RS: Pale greenish yellow SP: None
Inorganic salts-starch agar (ISP medium 4)	G: Good AM: Abundant, bluish gray RS: Dark blue to dark green SP: None	Sucrose-nitrate agar	G: Good AM: Moderate, white RS: Greenish gray SP: Pale orange
Tyrosine agar (ISP medium 7)	G: Good AM: Abundant, bluish gray RS: Dark brown SP: Yellowish brown	Glycerol-asparagine agar	G: Good AM: Abundant, bluish gray RS: Olive brown SP: Yellowish brown
Glucose-asparagine agar	G: Good AM: Abundant, bluish gray RS: Yellowish brown SP: Pale brown	Peptone-yeast extract-iron agar	G: Good AM: None RS: Light brown SP: Brown

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

GOTTLIEB[1], and WAKSMAN[2]. Incubation was carried out at 30°C for 21 days. The color names used in this study were taken from Methuen Handbook of Colour[3]. Results are shown in Table 1. The aerial mycelium was bluish gray. Reverse side of growth was brownish orange on yeast extract-malt extract agar, grayish green on oatmeal agar, dark blue to dark green on inorganic salts-starch agar. The pigment of the reverse mycelium was pH sensitive, changing from bluish or greenish to red or pink with the addition of 0.05 N HCl. Pale orange to brown soluble pigment was produced on several media.

Table 2. Physiological characteristics of strain No. 7587.

Temperature range for growth	15~38°C
Optimum temperature for growth	29~35°C
Liquefaction of gelatin	Positive
Coagulation of milk	Negative
Peptonization of milk	Positive
Hydrolysis of starch	Positive
Production of melanoid pigment	Positive
Decomposition of cellulose	Negative

Wall analysis was performed by the methods of BECKER *et al.*[4] and YAMAGUCHI[5]. Analysis of the whole cell hydrolysate showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Physiological properties of strain No. 7587 were as follows. Temperature range for growth was determined on yeast extract-malt extract agar. Summarized physiological properties of strain No. 7587 are shown in Table 2. Temperature range for growth was from 15 to 38°C with optimum temperature from 29 to 35°C. It showed the positive reaction in liquefaction of gelatin, peptonization of milk and starch hydrolysis and the negative reaction in coagulation of milk. Formation of melanoid pigment was observed on peptone-yeast extract-iron agar and in Tryptone-yeast extract broth.

Utilization of carbon was examined according to the method of PRIDHAM and GOTTLIEB[6]. The results were determined after 14 days incubation at 30°C and shown in Table 3.

The microscopic studies and cell wall composition of strain No. 7587 showed that it belongs to the genus *Streptomyces* Waksman and Henrici 1943. Accordingly, a comparison of this strain was made with published descriptions of various *Streptomyces* species. As a result, strain No. 7587 was considered

Table 3. Utilization of carbon sources by strain No. 7587.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	+
Mannitol	+
D-Fructose	+
L-Rhamnose	+
Sucrose	+
Raffinose	+

Table 4. Comparison of taxonomic characteristics of strain No. 7587 and *Streptomyces viridochromogenes* IFO 3113.

	No. 7587	IFO 3113
Aerial mass color	Bluish gray	Bluish gray
Melanoid pigment	Positive	Positive
Spore chain	Spiral	Spiral
Spore surface	Smooth	Smooth
Gelatin liquefaction	Positive	Positive
Milk peptonization	Positive	Positive
Starch hydrolysis	Positive	Weakly positive
Carbon source utilization		
Sucrose	+	+
D-Xylose	+	+
D-Fructose	+	+
L-Rhamnose	+	+
Raffinose	+	+
L-Arabinose	+	+
Inositol	+	+
Mannitol	+	+
Temperature range for growth (°C)	15~38	19~40

to closely resemble *Streptomyces viridochromogenes*. Therefore, strain No. 7587 was compared with *S. viridochromogenes* IFO 3113. As shown in Table 4, strain No. 7587 was recognized to be quite similar to *S. viridochromogenes* IFO 3113. The differences observed between the two organisms were starch hydrolysis and temperature range for growth. The differences, however, do not seem to us sufficient to make a distinction between the two organisms. Therefore, strain No. 7587 was identified as *S. viridochromogenes* and designated *S. viridochromogenes* No. 7587. It has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession No. FERM BP-1639.

Fermentation

A seed medium (160 ml) consisted of soluble starch 1%, sucrose 1%, glucose 1%, peptone 0.5%, soy bean flour 0.5%, cotton-seed flour 1% and calcium carbonate 0.1% was dispersed into each of six 500-ml Erlenmeyer flasks and sterilized. A loopful of slant culture of *S. viridochromogenes* No. 7587 was inoculated to each of the medium and cultured under shaking condition at 30°C for 3 days.

A production medium (20 liters) consisted of soluble starch 6%, dried yeast 1%, wheat germ 2%, calcium carbonate 0.3% and sodium iodide 0.001% was dispersed into each of three 30 liter-jar fermenters and sterilized.

The resultant seed culture broth (320 ml) was inoculated to the production medium and cultured at 30°C for 3 days, agitated at 200 rpm and aerated at 20 liters per minute. The amount of antibiotic produced was determined by a paper-disk agar diffusion method using *Escherichia coli* 8S-1 as the test organism.

Isolation

The procedure for purification of FR112123 is outlined in Fig. 2. The cultured broth (60 liters) was filtered with the aid of diatomaceous earth. The filtrate (27 liters) was passed through a column of a cation exchange resin, Diaion SK-1B (NH_4^+ type, 5 liters). The column was washed with water and eluted with 0.1 N ammonium hydroxide. The eluate (27 liters) was concentrated *in vacuo*. The residue was mixed with 300 ml of silica gel 60, then applied on a column (1 liter) of silica gel and developed with a solution of butanol - ethanol - chloroform - 28% aqueous ammonia (2 : 4 : 1 : 1). FR112123 was eluted in the fractions from 3.6 to 5.5 liters and concentrated under reduced pressure. The residue was dissolved in a solution of butanol - acetic acid - water (3 : 1 : 2, 5 ml) and subjected to column chromatography of silica gel (200 ml)

Fig. 2. Isolation procedure of FR112123.

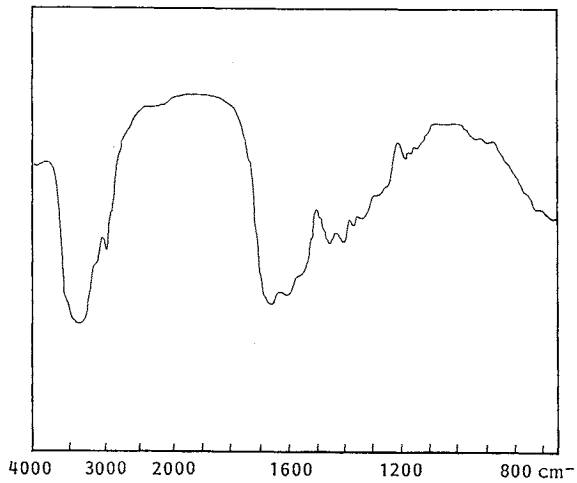
Culture filtrate (27 liters)
↓
SK-1B (NH_4^+)
↓ eluted with 0.1 N NH_4OH
Silica gel
↓ eluted with BuOH - EtOH - $CHCl_3$ - 28% aq ammonia (2 : 4 : 1 : 1)
Silica gel
↓ eluted with BuOH - AcOH - H_2O (3 : 1 : 2)
CM-Sephadex C-25 (NH_4^+)
↓ eluted with 0.02 N NH_4OH
FR112123 (410 mg)

Table 5. Physico-chemical properties of FR112123.

Molecular formula	$C_{16}N_{30}N_6O_6$
Anal for	$C_{16}H_{30}N_6O_6 \cdot 2H_2O$
Calcd:	C 43.82, H 7.82, N 19.17
Found:	C 43.20, H 7.57, N 18.97
MW (FAB-MS *m/z*)	403 $(M+H)^+$
$[\alpha]_D^{20}$ (MeOH)	$-5.7°$ (*c* 1.0)
UV $\lambda_{max}^{H_2O}$ nm (ε)	End
IR ν_{max} (KBr) cm^{-1}	3350, 2930, 1655, 1590, 1445, 1400, 1360, 1165
^{13}C NMR (100 MHz, D_2O) ppm	20.4, 24.8, 30.0, 30.9, 34.0, 35.9, 42.0, 45.7, 53.7, 56.2, 57.0, 175.9, 176.3, 177.8, 180.6, 182.6
Rf value[a] (I)	0.2
(II)	0.5

[a] Silica gel TLC (Merck 5715), solvent system (I) BuOH - AcOH - H_2O (3 : 1 : 2), (II) BuOH - EtOH - $CHCl_3$ - NH_4OH (4 : 8 : 2 : 3).

Fig. 3. IR spectrum of FR112123 (KBr).



packed with the same solvent system. After being eluted with the solvent the fractions containing FR112123 were collected and concentrated under reduced pressure to a volume of 200 ml. The resultant aqueous solution was passed through a column (100 ml) of CM-Sephadex C-25 (NH_4^+ type). The column was washed with water (300 ml) and eluted with 0.02 N ammonium hydroxide. The eluate (200 ml) was concentrated under reduced pressure to give a white powder of FR112123 (410 mg).

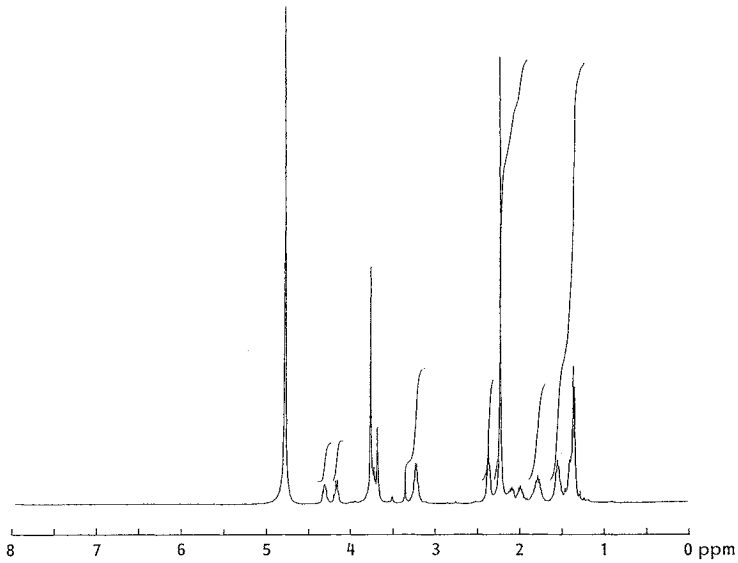
Physico-chemical Properties of FR112123

The physico-chemical properties of FR112123 (**1**) are summarized in Table 5. The compound was obtained as a white powder, soluble in water and methanol, but insoluble or sparingly soluble in acetone, ethyl acetate and chloroform. The elementary analysis, FAB-MS and ^{13}C NMR spectrum gave a molecular formula of $C_{16}H_{30}N_6O_6$. The IR and ^1H NMR spectra of **1** are shown in Figs. 3 and 4, respectively.

Structural Elucidation

The positive color reaction for ninhydrin and the presence of a band at 1655 cm^{-1} due to amide in

Fig. 4. ^1H NMR spectrum of FR112123 (400 MHz, D_2O).



the IR spectrum suggested that **1** was a peptide antibiotic. Therefore, the composition and sequence of amino acids were determined by the conventional methods of acid hydrolysis, hydrazine and Edman degradation.

Hydrolysis of **1** with 6 N HCl at 110°C for 20 hours in a sealed tube gave a mixture of glutamic acid, glycine, alanine, lysine and ammonia at a molar ratio of 1 : 1 : 1 : 1 : 1. These four amino acid residues account for all the carbon atoms required by the molecular formula of **1**. One mol of ammonia indicates that **1** possesses glutamine instead of glutamic acid residue. After derivatization with 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC), the hydrolysate was examined by reversed phase HPLC[7]. Comparison with authentic amino acids revealed at **1** had L-glutamine, D-alanine and D-lysine (see Experimental).

Hydrazine degradation of **1** revealed alanine as its *C*-terminal amino acid. *N*-Terminal amino acid of **1** was determined for glycine by Edman degradation.

From the results described above, it was deduced that the structure of **1** was a tetrapeptide consisted of L-Gln, Gly, D-Ala and D-Lys, which had glycine at the *N*-terminus and alanine at the *C*-terminus. Nevertheless, because of the presence of *γ*-carboxyl group in glutamine and *ε*-amino group in lysine, the sequence of these amino acids in the peptide could be speculated in many cases.

Therefore, further studies by NMR needed to be done. **1** was treated with di-*tert*-butyl dicarbonate ($(Boc)_2O$) and methylated with CH_2N_2. The ^1H NMR spectrum of Boc-FR112123-Me (**2**) thus obtained indicated that **2** had two Boc and one methyl group. The proton assignment of **2** was achieved by ^1H-^1H COSY and the results are shown in Table 6 with that of **1**. The amino acid sequence of **1** was determined by the spectral analysis of phase-sensitive NOESY[8]. Thus, the observation of NOE between Ala-NH and Lys-α CH indicates that the preceding amino acid of Ala is Lys. The linkage of Gln-α carbonyl and Lys-ε NH is verified by NOE between Gln-α CH and Lys-ε NH. Taking into account of molecular formula of **2** led us to connect the Gly carbonyl and Gln-α NH to give gross structure of **2**. On the basis of the results obtained so far, the structure of FR112123 was established as **1**.

Table 6. Assignment of the proton in ^1H-^1H COSY spectra of FR112123 (**1**) and Boc-FR112123-Me (**2**) (400 MHz).

		FR112123 (**1**) in D_2O[a]	Boc-FR112123-Me (**2**) in $CDCl_3$[a]
Gly	NH		6.15 (br)
	α CH_2	3.75 (2H, m)	3.75 (1H, m), 3.85 (1H, m)
Gln	NH		7.64 (br)
	α CH	4.31 (1H, m)	4.64 (1H, m)
	β CH_2	2.00 (1H, m), 2.10 (1H, m)	2.00 (1H, m), 2.15 (1H, m)
	γ CH_2	2.38 (2H,m)	2.30 (2H, m)
	$CONH_2$		6.40 (br), 6.85 (br)
Lys	NH		5.56 (1H, m)
	α CH	3.72 (1H, m)	4.15 (1H, m)
	β CH_2	1.78 (2H, m)	1.55 (1H, m), 1.74 (1H, m)
	γ CH_2	1.40 (2H, m)	1.35 (2H, m)
	δ CH_2	1.55 (2H, m)	1.45 (2H, m)
	ε CH_2	3.23 (2H, m)	3.14 (1H, m), 3.36 (1H, m)
	ε NH		7.75 (br)
Ala	NH		7.80 (br)
	α CH	4.18 (1H, m)	4.55 (1H, m)
	β CH_3	1.37 (3H, d)	1.35 (3H, d)
	$COOCH_3$		3.75 (3H, s)
	Boc		1.45 (9H, s), 1.46 (9H, s)

[a] δ (ppm), multiplicity.

Table 7. Antimicrobial spectrum of FR112123.

Test organism	MIC (μg/ml)
Escherichia coli 22	31
Klebsiella pneumoniae 1	>500
Proteus mirabilis 4	250
Staphylococcus aureus FDA 209P	>500
Micrococcus luteus	>500
Bacillus subtilis ATCC 6633	500
Candida albicans FP 633	>500
Acholeplasma laidlawii	>500

Mueller-Hinton agar was used as the assay medium.

Table 8. MIC values of FR112123 and other antibiotics against a mutant sensitive to inhibitors of cell wall synthesis.

Antibiotic	MIC (μg/ml)	
	Escherichia coli NIHJ	*E. coli* 8S-1[a]
FR112123	250	2
Cephalosporin C	>1,000	16
6-Aminopenicillanic acid	250	63
Nocardicin A	250	4
Vancomycin	500	2
Tetracycline	3.1	3.1
Erythromycin	50	100
Lincomycin	>100	>100

[a] A hyper-sensitive mutant of *E. coli* NIHJ.
MIC was measured by paper-disk agar diffusion method.

Biological Properties

Antimicrobial activity of FR112123 was measured by agar dilution method in a conventional manner. The results show that FR112123 has antimicrobial activity against a certain Gram-negative bacteria (Table 7). The MIC value of FR112123 against an *E. coli* mutant sensitive to inhibitors of cell wall synthesis is remarkably lowered as shown in Table 8. The mutant is hyper-sensitive to only the inhibitors which act on the various steps in biosynthesis of bacterial cell wall. Therefore, this result suggests that FR112123 also acts on the cell wall synthesis. Further study will be necessary to determine the precise mode of action.

Acute toxicity of FR112123 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

The structure of FR112123 resembles the partial structure of peptide in bacterial peptidoglycan.

Compared with -L-Ala-D-Glu$^{\gamma}$-*meso*-Dap-D-Ala in *E. coli* and -L-Ala-D-Glu$^{\gamma}$-L-Lys-D-Ala- in *Micrococcus lysodeikticus*, the structure of **1** different from those of bacterial peptide in two respects. First, the compound **1** has amino acids with inverse configuration from that of the amino acids in peptidoglycan. In the second place, Glu in peptidoglycan is replaced by Gln which is linked by a peptide bond to the ε-amino group of D-Lys. A hyper-sensitive mutant to inhibitors of cell wall biosynthesis is very susceptible to FR112123. These results suggest that FR112123 may act on the biosynthesis of intermediate in bacterial peptidoglycan.

Experimental

General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM400 spectrometer at 400.13 and 100.6 MHz, respectively. The chemical shifts were reported as ppm downfield from TMS. FAB-MS were measured by a VG ZAB SE mass spectrometer.

Determination of Absolute Configuration of Amino Acids

The GITC derivatives of acid hydrolysate of **1** were analyzed on reversed phase HPLC and their retention times are summarized in Table 9. HPLC condition: Column; TSK gel ODS-80TM 4.6 × 250 mm (Tosoh Manufacturing Co., Ltd.): Dual mobile phases; A = 0.1% H_3PO_4 - MeOH (9 : 1), B = MeCN - MeOH (9 : 1), linear gradient elution from 20% B to 40% B for 60 minutes.

Table 9. Retention times of GITC derivatives of acid hydrolysate of **1**.

	Authentic amino acid (minutes)	Hydrolysate of **1** (minutes)
Glu L	22.3	22.1
D	23.9	
Ala L	26.2	
D	29.4	29.2
Lys L	65.5	
D	66.3	66.5

Preparation of Boc-FR112123-Me (**2**)

To a solution of FR112123 (**1**, 40 mg) in H_2O (1 ml) containing Et_3N (56 μl) was added di-*tert*-butyl dicarbonate (88 mg), and the reaction mixture was stirred for 60 minutes at room temperature and evaporated to dryness. The residue was dissolved in MeOH (1 ml) and treated with excess CH_2N_2. After standing for 5 minutes, the mixture was evaporated to dryness to give an oil, which was purified by preparative TLC developing with 10% MeOH in $CHCl_3$ to give **2** as oil (26 mg).

Acknowledgments

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