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FR252921, a Novel Immunosuppressive Agent Isolated from *Pseudomonas fluorescens* No. 408813

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities of FR252921, FR252922 and FR256523

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Novel immunosuppressive agents, FR252921, FR252922 and FR256523 were isolated from the cultured broth of a bacterial strain No. 408813. The strain was identified *Pseudomonas fluorescens* from morphological and physiological characteristics.

FR252921, FR252922 and FR256523, novel compounds containing macrolactone ring, showed immunosuppressive activity against murine splenocyte proliferation stimulated with lipopolysaccharide (LPS) or anti-CD3 mAb *in vitro*.

In recent years, many new immunosuppressive drugs have been discovered and developed for clinical use in organ transplantation[1]. Almost all of patients are treated with calcineurin (CN) inhibitors based therapy, using cyclosporine A (CsA) or FK506, as part of dual, triple, or sequential therapy[2]. However, we have some problems to be resolved, including chronic rejection and side effect. To establish therapy with more efficacy and safety, we need another novel immunosuppressant that has different target from that of immunosuppressants used in clinical, especially CN inhibitors.

FK506 and CsA act by interaction with their cognate intracellular receptors, cyclophilin and FKBP, respectively. The Ca^{2+}/calmodulin-regulated phosphatase CN is a major target of drug-isomerase complexes. They prevent from transport of NF-AT into nucleus, leading to inability to produce IL-2[3,4]. So, FK506 dramatically inhibit T cell meditated immune response, such as mixed lymphocyte reaction and splenocyte proliferation stimulated anti-CD3 mAb[5,6]. On the other hand, many reports mentioned involvement of antigen presenting cell (APC) with graft rejection and suggested that APC will be important target cell population for transplantation therapy[7]. However, CN inhibitors are insufficient to suppress APC function. For example, LPS-mediated activation of APC, such as B cell and dendritic cell, is less sensitive to FK506 than T cell activation[8,9]. In order to find a novel drug that inhibits APC functions, we considered LPS-activated proliferation as useful *in vitro* assay.

In the course of searching for immunosuppressants, from soil microorganisms, FR252921 (**1**), FR252922 (**2**) and FR256523 (**3**) (Fig. 1) were isolated from the cultured broth of *Pseudomonas fluorescens* No. 408813. They inhibited proliferation of splenocyte stimulated with LPS and anti-CD3 mAb in contrast to their little toxic effect on non-specific proliferation of EL-4, lymphoma. This paper describes the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of these compounds.

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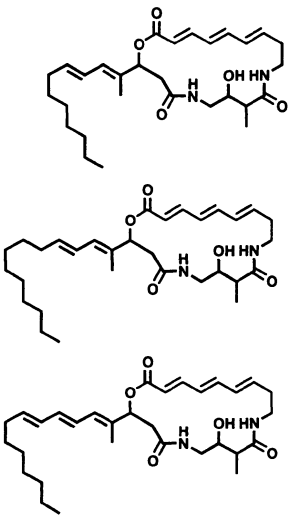
Fig. 1. Structures of FR252921, FR252922 and FR256523.



FR252921 (**1**)

FR252922 (**2**)

FR256523 (**3**)

Materials and Methods

Taxonomic Study

The taxonomic studies were based on the methods described in BERGEY's Manual of Systematic Bacteriology (Vol. 1)[10] and Manual for Identification of Medical Bacteria[11]. Morphological observation of strain No.408813 was carried out using a light microscope and a scanning electron microscope with cells grown on nutrient agar for 24 hours at 30°C.

Media Used for Seed Culture and Production

The seed medium consisted of Nutrient Broth (Kyokuto Seiyaku, Japan) 2%. The production medium was composed of glycerin 2.2%, modified starch 2.2%, Nutrient Broth 3.3%, soybean meal 3.3%, $CaCO_3$ 0.2%, Antifoam No. 8 (Kao Corporation, Japan) 0.125%.

HPLC Analysis

Detection of compounds **1**~**3** from the fermentation broth and the fractions under purification were monitored by HPLC using a reverse phase column YMC-ODS-AM (AM-302, 150×4.6 mm i.d., YMC Co., Ltd.). The mobile phase was 80% aqueous acetonitrile. The flow rate was 1.0 ml/minute. The detection wavelength was set at 210 nm.

Immunosuppressive and Cytotoxic Activity

As described previously, splenocytes stimulated with LPS or anti-CD3 mAb proliferate and produce various cytokines[12~15]. So, immunosuppressive activity was determined with those proliferation assay. Splenocytes was prepared from female C57BL/6 mice (Charles River Japan Inc.) and suspended in 0.1 ml RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Moregate, Bulimba, Australia), 50 mM 2-mercaptoethanol (Nakarai Chemical, Japan), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen, Rockville, MD). Proliferation assay was performed in 96-well U-bottomed microtiter plates with each well containing 1×10^5 splenocytes in 0.1 ml medium, to which anti-mouse CD3 mAb (1 μg/ml) or LPS (Sigma) (10 μg/ml) was added. The cells were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2. Proliferation was measured by the colorimetric MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide) assay described by MOSMANN[16]. Briefly, 4 hours prior to termination of culture, 10 μg of MTT dissolved in RPMI-1640 was added to the each well. After removal of the medium from all wells, 2-propanol was added to each well and mixed thoroughly to dissolve the dark blue crystals. The plates were measured on a two-wavelength microplate photometer at 550 nm with a reference wavelength at 660 nm.

In vitro cytotoxic activity was tested 96-well U-bottomed microtiter plates, with each well containing 1×10^3 murine lymphoma EL-4 cells in 0.1 ml medium. The cells were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2. Proliferation was measured by MTT assay as described above.

Results

Taxonomic Studies

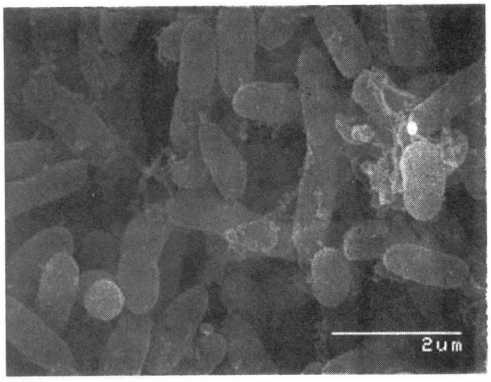
The bacterial strain No. 408813 was originally isolated from a soil sample collected in Nagano prefecture, Japan. Morphological observation of strain No. 408813 was carried out by a light microscope and an electron microscope with cells grown on nutrient agar for 24 hours at 30°C (Table 1 and Fig. 2). Strain No. 408813 was a Gram-negative, motile bacterium. Colonies of No. 408813 on nutrient agar were smooth, pale orange in color, circular and entire-edged. The spores were not formed. The cell shape was rod with a size of 0.8~1.0×2.0~3.0 μm.

Physiological characteristics of strain No. 408813 were summarized in Table 2. The growth temperature was from 3°C to 32°C. Strain No. 408813 gave positive results in test for catalase, oxidase, Simmons citrate, urease, tween 80 hydrolysis, gelatin liquefaction, and arginine dihydrolase.

Table 1. Morphological characteristic of strain No. 408813.

Gram stain	Negative
Color of colony	Pale orange
Cell shape	Rod
Cell size	0.8-1 x 2-3 μm
Motility	Positive
Spore formation	Negative

Fig. 2. Electron micrograph of strain No. 408813.



Scale: 2 μm.

Strain No. 408813 was O-F test oxidative and pyoverdin production positive. Nitrate reduction, esculin hydrolysis, starch hydrolysis, ONPG test and Dnase were negative.

Acid formation was observed from D-glucose, D-xylose and D-mannitol. The following compounds were utilized as a sole carbon source: namely, D-glucose, D-mannose, D-xylose, D-mannitol, D-trehalose, glycerin, sucrose, maltose, *N*-acetyl-D-glucosamine, gluconate, caprate and malate.

According to BERGEY's Manual of Systematic Bacteriology (Vol. 1)[11], strain No. 408813 was considered to belong to genus *Pseudomonas* from those characteristics described above. Thus, strain No. 408813 was compared with *Pseudomonas* species described in literature. As a result, it was found that the strain proved to closely resemble *Pseudomonas fluorescens*. Therefore, strain No. 408813 was identified as *Pseudomonas fluorescens*.

Table 2. Physiological characteristics of strain No. 408813.

Growth temperature	3 - 32 ℃
Optimum growth temperature	5 - 30 ℃
Growth in air	Positive
Growth on MacConkey	Positive
Pyoverdin production	Positive
Catalase	Positive
Oxidase	Positive
O-F test	Oxidative
Simmons citrate	Positive
Nitrate reduction	Negative
Indole	Negative
H2S (SIM)	Negative
Esculin hydrolysis	Negative
Starch hydrolysis	Negative
ONPG test	Negative
DNase	Negative
Urease	Positive
Tween 80 hydrolysis	Positive
Gelatin liquefaction	Positive
Casein hydrolysis	Negative
Lysine decarboxylase	Negative
Arginine dihydrolase	Positive
Ornitine decarboxylase	Negative
Acid formation from	
D-Glucose	Positive
D-Xylose	Positive
D-Mannitol	Positive
Lactose	Negative
Sucrose	Negative
Maltose	Negative
Utilization of	
D-Glucose	Positive
L-Arabinose	Negative
D-mannose	Positive
D-Xylose	Positive
D-Mannitol	Positive
D-Trehalose	Positive
Glycerin	Positive
Lactose	Negative
Sucrose	Positive
Maltose	Positive
N-Acetyl-D-glucosamine	Positive
Gluconate	Positive
Caprate	Positive
Adipate	Negative
Malate	Positive
Phenylacetate	Negative

Fermentation

A slant culture of the producing strain was inoculated into a 225-ml Erlenmeyer flask containing 60 ml of the seed medium. After incubation at 30°C for 24 hours on a rotary shaker (220 rpm), 1.2 ml of the culture was inoculated into a 225-ml Erlenmeyer flask containing 60 ml of the same

seed medium, and incubated at 30°C for 24 hours on a rotary shaker (220 rpm). Four hundreds milliliters of the resultant seed culture was transferred into a 30-liter jar fermentor. The fermentation was carried out at 25°C for 3 days under aeration of 20 liters/minute and agitation of 200 rpm.

Isolation and Purification

After the culture was completed, the culture broth was extracted with an equal volume of acetone by stirring for a few minutes at room temperature. The extract was filtered with an aid of diatomaceous earth. The filtrate was extracted with 36 liters of *n*-hexane/ethyl acetate (1 : 1) solution. The solvent extract (upper layer) was concentrated under reduced pressure to an oily residue. This residue was dissolved in 500 ml of acetonitrile and washed three times with equal volume of *n*-hexane. Then the acetonitrile layer was concentrated under reduced pressure. The residue was dissolved in 50 ml of methanol and soon passed through a column (1 liter) of Daisogel SP-120-ODS-B (15/30 μm, DAISO Co., Ltd., Japan) packed with 40% aqueous acetonitrile. The column was eluted with 65% aqueous acetonitrile (6 liters, **1**), 70% aqueous acetonitrile (4 liters, **3** and **2**) and 75% aqueous acetonitrile (1.5 liters, **2**). The elution was monitored by analytical HPLC. Fractions containing **1**, **2** and **3** were combined respectively. The portion corresponding to **1** was extracted with equal volume of *n*-hexane/ethyl acetate (1 : 2) solution. The solvent extract (upper layer) was concentrated under reduced pressure to a powder. The powder was washed with acetonitrile, filtered and dried up to give 175 mg of **1** as a white powder.

The portion corresponding to **3** was extracted with equal volume of *n*-hexane/ethyl acetate (1 : 2) solution. The solvent extract (upper layer) was concentrated under reduced pressure to a powder. The powder was washed with acetonitrile, filtered and dried up to give 74 mg of **3** as a white powder.

The portion corresponding to **2** was extracted with equal volume of *n*-hexane/ethyl acetate (1 : 2) solution. The solvent extract was concentrated under reduced pressure to a powder. This powder was dissolved in 50 ml of methanol and soon passed through a column (1 liter) of Daisogel SP-120-ODS-B packed with 40% aqueous acetonitrile. The column was eluted with 75% aqueous acetonitrile. Fractions containing **2** were combined. And then, **2** was extracted with equal volume of *n*-hexane/ethyl acetate (1 : 2) solution. The solvent extract (upper layer) was concentrated under reduced pressure to a powder. The powder was washed with acetonitrile, filtered and dried up to give 79 mg of **2** as a white powder.

Physico-chemical Properties

As shown in Table 3, compounds **1**, **2** and **3** were soluble in chloroform and dimethyl sulfoxide, slightly soluble in acetonitrile and methanol but insoluble in *n*-hexane and water. They show positive color reactions to iodine vapor and Dragendorf, though negative against Morish, Ahrlich, $FeCl_3$ and Ninhydrin. They showed UV absorption at 239 and 304 nm. The ES-MS spectrum showed a molecular ion peak at *m/z* 501, 529 and 527 ($M+H^+$, **1**, **2** and **3** respectively). The ^1H and ^{13}C NMR spectra of **1** are shown in Fig. 3 and Fig. 4.

The determinations of the structures were accomplished primarily by a series of 2-D NMR techniques. Detailed of the structure elucidation studies of **1**, **2** and **3** will be described elsewhere.

In Vitro Immunosuppressive Activity and Cytotoxic Activity

The *in vitro* immunosuppressive activities of **1**, **2** and **3** were analyzed using lymphocyte growth inhibition assay. In this study, the immunosuppressive effect was calculated by reduction in lymphocyte proliferation quantified as strength of staining by MTT. **1**, **2** and **3** inhibited proliferation stimulated with LPS, comparably insensitive to FK506 or CsA. Moreover, **1**, **2** and **3** also inhibited splenocyte proliferation stimulated with anti-CD3 mAb with comparable IC_{50} value to CN inhibitors, FK506 or CsA (Table 4). Similar inhibitory effects of them on mixed lymphocyte reaction were confirmed (data not shown).

The cytotoxic activities of **1**, **2** and **3** against EL-4, murine lymphoma cell line were analyzed. Their IC_{50} were 641, 146 and 760 ng/ml, respectively.

Discussion

Compounds **1**, **2** and **3**, which exhibited immunosuppressive activities, were isolated from the culture broth of *Pseudomonas fluorescens* No. 408813. From the evidence of physico-chemical data, these compounds were to have novel structures (Fig. 1). Compounds that have 19-members macrolactone ring containing two amide bonds are rarely found in nature. Thus, our screening system with LPS-activation assay and cytotoxic assay is useful to find novel immunosuppressant different from CN inhibitor.

Table 3. Physico-chemical properties of compound **1**~**3**.

	1	**2**	**3**
Appearance	white powder	white powder	white powder
MP	140~144℃	142~146℃	88~93℃
$[a]^{23}_{D}$	−222° (*c* 0.2, DMSO)	−235° (*c* 0.2, DMSO)	−72° (*c* 0.3, DMSO)
ES-MS (*m/z*)	501 $(M+H)^+$	529 $(M+H)^+$	527 $(M+H)^+$
Molecular formula	$C_{29}H_{44}N_2O_5$	$C_{31}H_{48}N_2O_5$	$C_{31}H_{46}N_2O_5$
Elemental analysis			
Calcd for $C_{29}H_{44}N_2O_5$:	C 67.15, H 8.94, N 5.40	C 70.42, H 9.15, N 5.30	
Found:	C 66.56, H 8.69, N 5.30	C 70.13, H 9.29, N 5.24	
UV $\lambda_{max}^{acetonitorile}$ nm (ε)	239 (32200), 304 (39700)	239 (29300), 304 (36700)	266 (sh), 278 (60700), 291 (59400)
Color test			
Positive	I_2, $Ce(SO_4)_2$-H_2SO4, Dragendorf	I_2, $Ce(SO_4)_2$-H_2SO4, Dragendorf	I_2, $Ce(SO_4)_2$-H_2SO4, Dragendorf
Negative	$FeCl_3$, Morish, Ninhydrin, Ahrlich	$FeCl_3$, Morish, Ninhydrin, Ahrlich	$FeCl_3$, Morish, Ninhydrin, Ahrlich
Solubility			
Soluble	CH_3Cl, DMSO, DMF, THF	CH_3Cl, DMSO, DMF, THF	CH_3Cl, DMSO, DMF, THF
Slightly soluble	acetone, mehtanol, acetonitrile	acetone, mehtanol, acetonitrile	acetone, mehtanol, acetonitrile
Insoluble	H_2O, n-hexane	H_2O, n-hexane	H_2O, n-hexane
IR ν max (KBr) cm^{-1}	3300, 2930, 1710, 1640, 1620, 1540, 1380, 1250, 1230, 1060, 1005, 970	3300, 2920, 1710, 1650, 1620, 1540, 1250, 1230, 1060, 1000, 970	3300, 2930, 1720, 1650, 1540, 1260, 1100, 1005
TLC (Rf value)			
System I[a]	0.55	0.55	0.55
System II[b]	0.50	0.39	0.43

[a] Plate: Silica gel 60 F_{254} (E. Merck Co.), $CHCl_3$:CH_3OH = 10:1.

[b] Plate: RP-18 WF_{254} (E. Merck Co.), 80% aq. Acetonitrile.

Fig. 3. 400 MHz ^1H NMR spectrum of **1** in CD_2Cl_2.

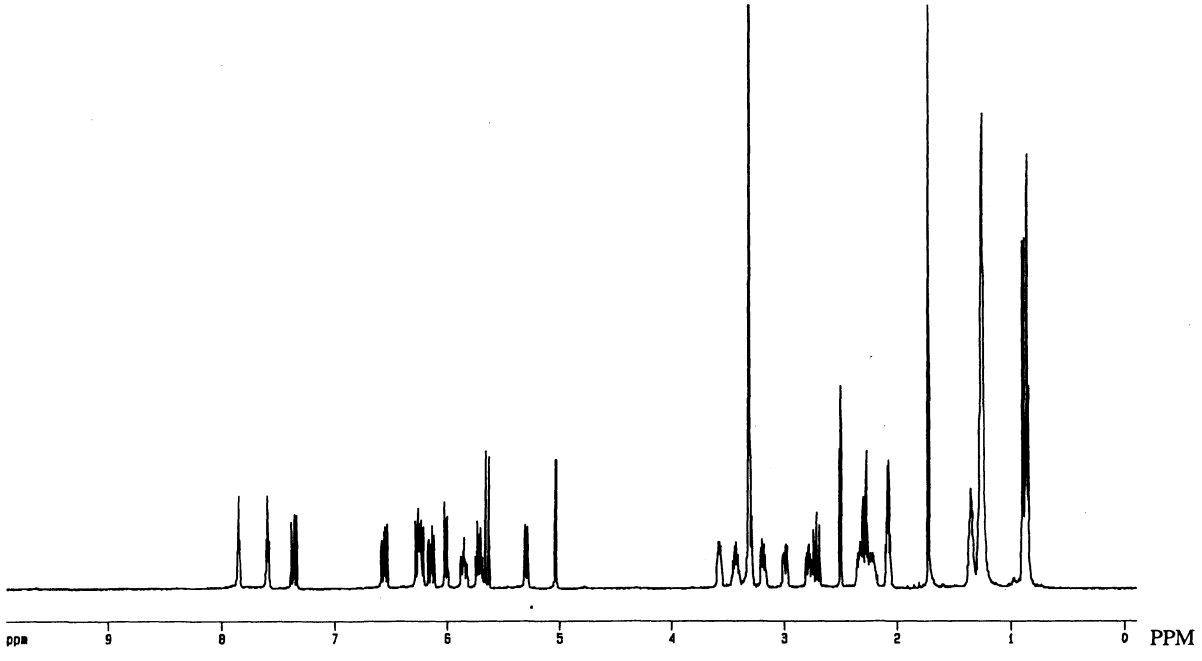


Fig. 4. 100 MHz ^{13}C NMR spectrum of **1** in CD_2Cl_2.

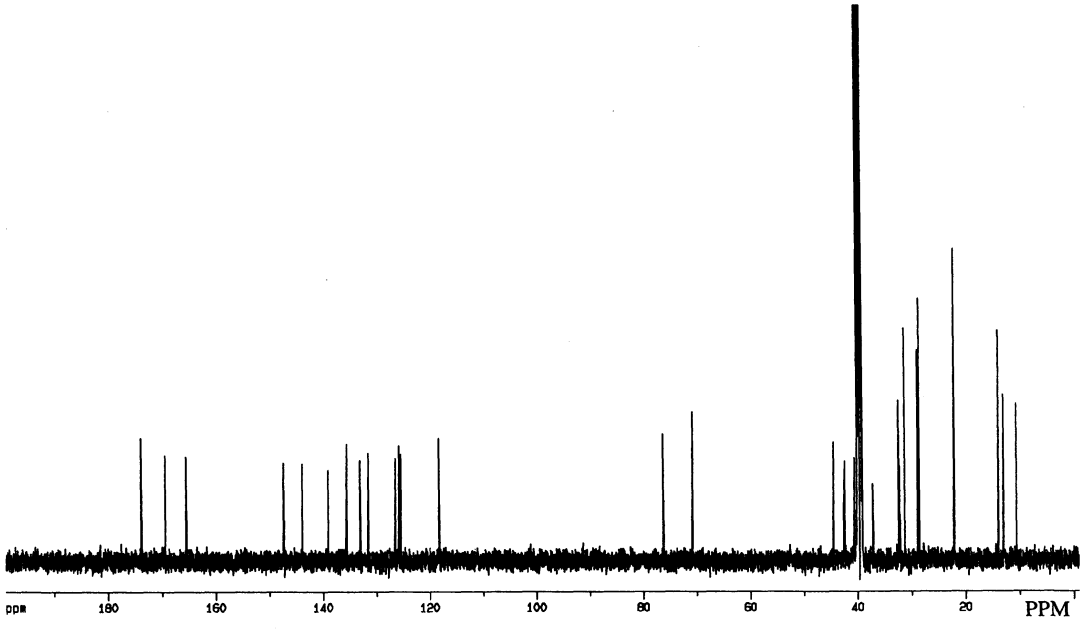


Table 4. IC_{50} values of **1**, **2**, **3**, FK506 and CsA against murine splenic proliferation and cytotoxic activity *in vitro* (ng/ml).

Cell	Splenocyte	Splenocyte	EL-4
Stimulus	Anti-CD3 mAb	LPS	
Drug			
1	10.9	3.5	641
2	3.9	2.9	146
3	39.7	21.7	760
FK506	0.2	34.0	>1000
CsA	2.5	28.7	>1000

Compounds **1**, **2** and **3** inhibited the lymphocyte proliferation stimulated with LPS and anti-CD3 in contrast to their little toxic effect on non-specific proliferation of EL-4. On the other hand, FK506 and CsA, T cell specific immunosuppressant, showed strong inhibition of splenocyte proliferation stimulated with anti-CD3 mAb, in contrast to little effect on proliferation stimulated with LPS. These results strongly suggest that target of **1**, **2** and **3** is different from a target of FK506 and CsA, and may facilitate further studies on the signaling pathway of LPS-mediated activation.

The details of *in vivo* evaluation and the mode of action of these compounds are described in the following papers[17,18]. The structural analysis will be described elsewhere.

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