J. Antibiot. 58(10): 634–639, 2005

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

FR177391, A New Anti-hyperlipidemic Agent from *Serratia*

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties, Structure Elucidation and Biological Activities

Bunji Sato[†], Hidenori Nakajima[†], Takashi Fujita[†], Shigehiro Takase[†], Seiji Yoshimura[†], Takayoshi Kinoshita[††], Hiroshi Terano[†††]

Received: March 29, 2005 / Accepted: October 6, 2005

Abstract In the course of screening for a new anti-hyperlipidemic agent from microbial products, we found that FR177391, produced by *Serratia liquefaciens* No. 1821, alleviated the decrease in lipid droplet formation in differentiated 3T3-L1 adipocyte cells, induced by the addition of tumor necrosis factor-α. Structural elucidation by spectroscopic methods and X-ray crystallographic analysis of its propylamide derivative revealed that FR177391 was a chlorinated macrocyclic lactone.

Keywords FR177391, hyperlipidemia, *Serratia*

Introduction

Elevated plasma lipid levels, especially triglyceride (TG) levels, are increasingly recognized as a risk factor for cardiovascular disease, and also commonly embedded in the context of metabolic syndromes, including obesity, diabetes and hypertension. Several classes of pharmacological agents aiming at improving hypertriglyceridemia have been developed. Adipocytes are known to play a key role in providing energy in times of demand and the storage of energy in the form of TG-rich lipid droplets. The differentiation of preadipocytes into mature triglyceride-containing adipocytes, a process termed adipogenesis, has been studied using the murine fibroblast-like preadipocyte cell line, 3T3-L1 [1～3]. When 3T3-L1 cells differentiate into mature lipid droplet-containing adipocytes, they assume a rounded morphology and form lipid droplets recognized by microscopic observation.

Up-regulation of tumor necrosis factor-α (TNF-α) level is thought to be a key factor in hypertriglyceridemia, insulin resistance and diabetes that often accompany obesity [4, 5]. As TNF-α is also known to inhibit the adipogenesis in 3T3-L1 fibroblasts [6], we designed a screening system for anti-hyperlipidemic agents using 3T3-L1 fibroblasts and TNF-α. In this system, compounds which break the inhibition of adipogenesis induced by TNF-α were selected. In the course of the screening for anti-hyperlipidemic agents from microbial products using this system, we found that *Serratia liquefaciens* No. 1821 produced a compound, FR177391, which alleviated the decrease in lipid droplet formation in differentiated 3T3-L1 adipocyte cells, induced by the addition of TNF-α [7]. In this paper, we describe the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of FR177391.

B. Sato (Corresponding author)**, H. Nakajima, T. Fujita, S. Takase, S. Yoshimura, T. Kinoshita, H. Terano:** Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-3, 5-chome, Tokodai, Tsukuba, Ibaraki 300-2698, Japan.
E-mail: bunji.sato@jp.astellas.com

[†] Present address: Astellas Pharma. Inc., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan
[††] Present address: Osaka Prefecture University, Graduate school of Science, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan
[†††] Present address: Toyo University, Faculty of Life Sciences, Itakura, Oragun, Gunma 374-0193, Japan

Materials and Methods

Microorganism and Taxonomy

The producing strain No. 1821 was isolated from a soil sample collected in New Zealand. The morphological, cultural and physiological characterization was carried out by the method described in Bergey's Manual of Systematic Bacteriology, 9th edition.

Culture and Medium Conditions

A loopful of bacterial strain No. 1821 grown on an agar slant was inoculated into fifteen 500-ml Erlenmeyer flasks containing 120 ml of a seed medium consisting of polypeptone 1%, yeast extract 0.5% and NaCl 0.5%. The flask was incubated in a rotary shaker (220 rpm) at 30°C for 1 day. The entire culture was transferred to five 30-liter jar fermenters (three flasks for each jar) containing 20 liters of production medium comprising glycerol 2%, glucose 1%, corn steep liquor 1%, defatted soybean powder 1%, meat extract 0.2%, ammonium sulfate 0.2%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.2%, Adecanol LG-109 (Asahi Denka) 0.05% and Silicon KM-70 (Shin-Etsu Kagaku) 0.05%. Cultivation was carried out at 25°C for 3 days at 200 rpm, one atom inner pressure and 20 liters/minute of an aeration volume.

Assay for FR177391 Production

The amount of FR177391 in the fermentation broth was determined by HPLC using YMC AM-303, S-5, A-120 column (4.6 mm inner diameter×250 mm length, YMC Co., Ltd.) at 210 nm with a mobile phase of 35% aqueous acetonitrile -50 mM phosphate buffer (pH 7.0) and at a flow rate of 1 ml/minute. Retention time of FR177391 was 6.7 minutes.

Antimicrobial Test

Antimicrobial activities of FR177391 were determined on Nutrient agar by a two-fold serial dilution method for bacteria. The lowest concentration that inhibited growth of bacteria after 18 hours incubation at 37°C is expressed in terms of μg/ml as the minimum inhibitory concentration (MIC).

Lipid Droplet Formation Assay

3T3-L1 (ATCC CL-173) cells were cultured in the growth medium composed of D-MEM (Sigma) medium with supplement of 10% Fetal calf serum and penicillin-streptomycin. On day 0, 2×10^4 of 3T3-L1 cells/well were seeded on 96-well plates. On day 2, the culture medium was changed to the induction medium (growth medium containing insulin (5 μg/ml; Sigma), 3-isobutyl-1-methylxanthine (0.5 mM; Sigma) and dexamethazone (1 μM; Sigma)) to induce differentiation. On day 4, the test compound dissolved in methanol was added to the medium (final 1% (v/v)) in the presence of TNF-α (2 ng/ml, Sigma), which is known to inhibit the lipid droplet formation. On day 8, lipid droplet formation was judged by the morphological change [8~10]. The lowest concentration of the test compound, where the lipid droplet formation was alleviated, was calculated by using the 2-fold serial dilution of the test compound. Methanol (final 1%(v/v)) didn't affect the lipid droplet formation.

Animals

BDF_1 mice (female, 6 weeks old) were purchased from Charles River Japan Inc., Atsugi, Japan.

Analytical Measurement

Optical rotation was measured with a Jasco DIP-140 polarimeter using a 10-cm microcell. UV spectrum was obtained with a Hitachi 220A spectrometer. The mass spectrum was recorded with VG ZAB-SE mass spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were acquired with a Bruker AM400wb spectrometer controlled with an ASPECT 3000 computer.

X-Ray Crystallographic Analysis

A colorless prismatic crystal of $C_{26}H_{38}NO_7Cl$ having approximate dimensions of 0.20×0.20×0.20 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and a rotating anode generator. Cell constants and an orientation matrix for data collection, obtained from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $50.46<\theta<58.88°$ corresponded to a primitive monoclinic cell with dimensions: a=13.499(1) Å, b=10.071(1) Å, c=10.604(1) Å, β=105.991(8)°, V=1385.8(3) Å^3. The calculated density is 1.60 g/cm^3 for Z=2 and F.W.=512.04. Based on the systematic absences of: 0k0: k±2n packing considerations, a statistical analysis of intensity distribution, and the successful solution and refinement of the structure, the space group was determined to be $P2_1$(#4). The data were collected at a temperature of 25±1°C using the ω-2θ scan technique to a maximum 2θ value of 136.0°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.29° with a take-off angle of 6.0°. Scans of $(1.00+0.30\tan\theta)°$ were made at a speed of 8.0°/minute (in omega). The weak reflections ($1<10.0\sigma(1)$) were rescanned (maximum of 3 scans) and the counts were

accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak to background counting time was 2 : 1. The diameter of the incident beam collimator was 0.3 mm and the crystal to detector distance was 235 nm. The computer-controlled slits were set to 3.0 mm (horizontal) and 3.0 mm (vertical).

Results

Taxonomy of the Producing Strain

Morphological observation of strain No. 1821 was made by the optical microscope with cells grown on nutrient agar at 30°C for 24 hours (Table 1). Strain No. 1821 was a Gram-negative, mobile bacilli. Colonies of No. 1821 on nutrient agar were smooth, pale orange in color, circular and entire-edged. No spore was formed. The cell shapes were rod with a size of 0.5~0.8×1.0~2.0 μm.

Physiological characteristics are shown in Table 2. The growth temperature range was from 3 to 34°C. Strain No. 1821 was positive for catalase, lysine decarboxylase, utilization of citrate, reduction of nitrate, ONPG test, VP test, and was fermentative for O-F test. This strain was positive for decomposition of gelatin, Tween80, casein and esculin, but negative for oxidase, decomposition of starch, and indole production. Acid formation was observed from D-glucose, D-xylose, D-fructose, D-mannitol, D-sorbitol, D-mannose, D-trehalose, glycerol, inositol, sucrose and salicin. This strain utilized D-glucose, D-xylose, D-fructose, D-mannitol, D-sorbitol, D-mannose, D-trehalose, glycerol, inositol, sucrose and maltose.

According to Bergey's Manual of Systematic Bacteriology, the strain was considered to belong to genus Serratia. So, strain No. 1821 was compared with *Serratia* species described in literature. Detailed examination suggested that this strain resembled *Serratia liquefaciens*. Comparison between *Serratia liquefaciens* JCM-1245 and strain No. 1821 showed no significant difference. Thus, this strain was identified as *Serratia liquefaciens* No. 1821.

Table 1 Cultural characteristics of strain No. 1821

Gram stain	Negative
Color tone of colony	Pale orange
Cell morphology	Bacillus
Cell size	0.5~0.8×1.0~2.0 μm
Mobility	Positive
Sporulation	Negative

Table 2 Physiological characteristics of strain No. 1821

Growth temperature	3~34°C	D-Fructose	Positive
Growth in air	Positive	D-Mannitol	Positive
Growth on MacConkey's agar medium	Positive	D-Sorbitol	Positive
Catalase	Positive	D-Mannose	Positive
Oxidase	Negative	D-Trehalose	Positive
O-F test	Fermentative	Glycerol	Positive
Utilization of citric acid	Positive	Inositol	Positive
Reduction of nitrate	Positive	Sucrose	Positive
Indole production	Negative	Lactose	Negative
H2S production (SIM)	Negative	Salicin	Positive
Esculin hydrolysis	Positive	Utilization of	
Starch hydrolysis	Negative	D-Glucose	Positive
ONPG test	Positive	D-Xylose	Positive
VP test	Positive	D-Fructose	Positive
DNase	Positive	D-Mannitol	Positive
Tween 80 hydrolysis	Positive	D-Sorbitol	Positive
Gelatin hydrolysis	Positive	D-Mannose	Positive
Casein hydrolysis	Positive	D-Trehalose	Positive
Lysine decarboxylase	Positive	Glycerol	Positive
Arginine dihydrolase	Negative	Inositol	Positive
Ornithine decarboxylase	Negative	Lactose	Negative
Acid production from		Sucrose	Positive
D-Glucose	Positive	Salicin	Negative
D-Xylose	Positive	Maltose	Positive

Fermentation of FR177391

Figure 1 shows a typical time course of FR177391 production by *Serratia liquefaciens* No. 1821 in a 30-liter jar fermenter, along with the pH of the medium. The production of FR177391 started at the second day and peaked at the third day. The amount of FR177391 in the fermentation broth reached about 1.6 μg/ml at the third day.

Isolation

The fermentation broth (100 liters) was extracted with 100 liters of acetone and was filtered with diatomaceous earth. The pH of the filtrate was adjusted to 7.0 with 6 N-NaOH and then extracted twice with 100 liters of ethyl acetate. The extracts were combined for concentration under reduced pressure. The concentrate (30 g) was applied to a 1 liter silica gel column, which was prefilled with *n*-hexane. After washing with 3 liters each of *n*-hexane, *n*-hexane - ethyl acetate (1 : 1), ethyl acetate, ethyl acetate - acetone (1 : 1) and acetone, the column was eluted with 3 liters of acetone - methanol (1 : 1). The acetone - methanol fraction was concentrated under reduced pressure, and the resulting oil (2.6 g) was applied to a 100 ml of silica gel column, which was prefilled with dichloromethane - methanol (25 : 1). After washing with 300 ml of dichloromethane - methanol (25 : 1) and dichloromethane - methanol (10 : 1), the column was eluted with 300 ml of dichloromethane - methanol (5 : 1) and 300 ml of dichloromethane - methanol (2 : 1). The active fractions were combined for concentration under reduced pressure. The active oily substance (180 mg) was dissolved in 10 ml of methanol, and applied on a 350 ml of ODS gel (YMC ODS-AM 120-S50) column. The column was eluted with 35% aqueous methanol - 50 mM phosphate buffer (pH 7.0). The active fractions were combined, extracted twice with ethyl acetate and concentrated under reduced pressure. The resulting crude powder (102 mg) was applied on an HPLC column (YMC ODS-AM 120-S5; i.d. 250×4.6 mm). The column was eluted with 35% aqueous acetonitrile - 50 mM phosphate buffer (pH 7.0). The active fractions were concentrated to remove acetonitrile and extracted with ethyl acetate three times. The extract was concentrated under reduced pressure to obtain a colorless powder of FR177391 (25 mg).

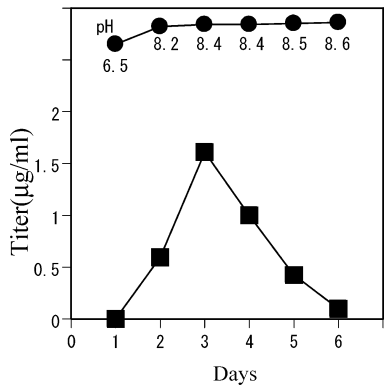


Fig. 1 Time course of FR177391 production (■) and pH change (●).

Physico-chemical Properties

FR177391 is acidic in nature, and showed positive color reaction to iodine vapor and ceric sulfate though negative to ninhydrin. It is soluble in methanol, acetone and chloroform but insoluble in hexane. The physico-chemical properties are listed in Table 3. According to high-resolution FAB-MS spectrometry, the molecular formula is $C_{23}H_{31}ClO_8$. In the ^{13}C NMR spectrum, all the ^{13}C signals were consistent with the carbon backbone presented in Fig. 2 (**1**). The ^{1}H and ^{13}C NMR spectral data of **1** were very similar to those of heterumalide NA [11, data not shown].

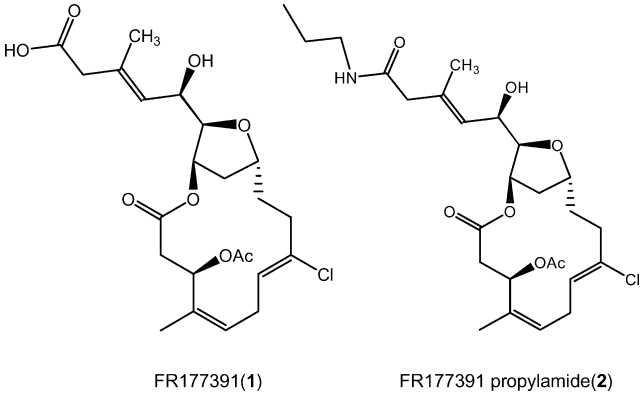
X-Ray Crystallographic Analysis of Propylamide Derivative of FR177391

In the course of the efforts to examine the structure-activity relationships of FR177391, its propylamide (**2**) (Fig. 2) was prepared by treatment of FR177391 with EDC, HOBt and propylamine (quantitative yield). Fortunately, the propylamide could be crystallized from methanol to give fine crystals suitable for X-ray crystallography. ORTEP drawing of the derivative **2** is shown in Fig. 3. Thus, the relative stereochemistry of FR177391 (**1**) was determined as shown in Fig. 2.

Table 3 Physico-chemical properties of FR177391

Appearance	Pale yellow powder
Melting Point (°C)	56～72
$[\alpha]_D^{32}$	+32° (*c* 0.85, MeOH)
Molecular formula	$C_{23}H_{31}ClO_8$
Molecular weight	
FAB-MS (*m/z*)	547 $(M+2K-H)^+$
UV λ_{max}^{MeOH} nm	206, 230 (sh)
Color test	
Positive	$Ce(SO_4)_2$, I_2
Negative	Ninhydrin, Molish, $FeCl_3$
Solubility	
Soluble	MeOH, $CHCl_3$, $(CH_3)_2CO$
Slightly soluble	EtOAc, H_2O
Insoluble	*n*-Hexane
TLC (Rf value)	
System I[a]	0.50

[a] Plate: Silica gel $60F_{254}$ (E. Merck Co.), CH_2Cl_2 : MeOH=2 : 1



FR177391(**1**) FR177391 propylamide(**2**)

Fig. 2 Chemical structures of FR177391 (**1**) and its propylamide derivative (**2**).

Biological Properties

FR177391 alleviated the decrease of lipid droplet formation of 3T3-L1 induced by TNF-α. Minimum alleviating activity was observed at 0.1 μM. Detailed description of the biological activity *in vitro* and the mechanism of action of FR177391 using chemical genetic approaches will be described in another paper [10].

Antimicrobial Activity

FR177391 was devoid of antimicrobial activity when tested *vs.* the following microorganisms at 1000 μg/ml; *Escherichia coli* NIHJ JC-2, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* 209P.

Acute Toxicity

The acute toxicity of FR177391 was determined in mice by a single intraperitoneal injection of graded doses of FR177391 in the distilled water containing 20% polyethylene glycol #400. The LD_{50} was 1 mg/kg.

Discussion

FR177391 was isolated from a fermentation broth of *Serratia liquefaciens* No. 1821 as a substance which alleviated the decrease in lipid droplet formation in differentiated 3T3-L1 adipocyte cells induced by the addition of TNF-α. Detailed pharmacological activities of FR177391 *in vivo* will be discussed in the accompanying paper [12]. Structural elucidation by propylamide derivatization and X-ray crystallographic analysis revealed that FR177391 has the relative stereochemistry shown in Fig. 2. The structure has been reported as those of haterumalide NA [11] and oocydin A [13]. However, the optical rotation of FR177391 (+32 : c0.85, MeOH) was distinct from those of haterumalide NA (−3.0 : c0.053, MeOH) and oocydin A (+18.2 : c0.22, MeOH), indicating that the absolute structure of FR177391 is different from those of haterumalide NA and oocydin A.

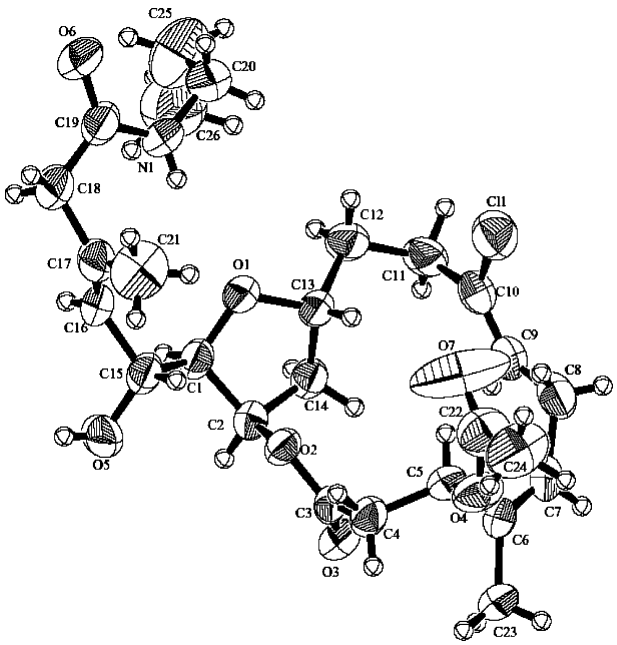


Fig. 3 ORTEP drawing of **2**.

Acknowledgements We are deeply grateful to Dr. H. Kigoshi (Tsukuba Univ.) for his useful advice and experiments for optical rotation.

References

1. Green H, Kahinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting adipose conversion. Cell 5: 19–27 (1975)
2. MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64: 345–373 (1995)
3. Ntambi JM, Young-Cheul K. Adipocyte differentiation and gene expression. J Nutr 130: 3122S–3126S (2000)
4. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259: 87–91 (1993)
5. Mohrschladt MF, Weverling-Rijnsburger AW, de Man FH, Stoeken DJ, Sturk A, Smelt AH, Westendorp RG. Hyperlipoproteinemia affects cytokine production in whole blood samples *ex vivo*. The influence of lipid-lowering therapy. Atherosclerosis 148(2): 413–419 (2000)
6. Kawakami M, Murase T, Ogawa H, Ishibashi S, Mori N, Takaku F, Shibata S. Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1

cells. J Biochem 101(2): 331–338 (1987)
7. Sato B, Ito K, Fujita T, Takase S, Terano H (Fujisawa). Therapeutic substances FR177391 for cancer cachexy. Japanese Pat. Appl. JP08-92272, April 9 (1996)
8. Souza SC, Palmer HJ, Kang YH, Yamamoto MT, Muliro KV, Eric Paulson K, Greenberg AS. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. J Cell Biochem 89: 1077–1086 (2003)
9. Stephens JM, Butts M, Stone R, Pekala PH, Bernlohr DA. Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipocyte differentiation by antagonists of adipogenesis. Cell Biochem 123: 63–71 (1993)
10. Yamaoka M, Sato K, Kobayashi M, Nishio N, Ohkubo M, Fujii T, Nakajima H. FR177391, a new anti-hyperlipidemic agent from *Serratia*. IV. Target identification and validation by chemical genetic approaches. J Antibiot 58: 654–662 (2005)
11. Takada N, Sato H, Suenaga K, Arimoto H, Yamada K, Ueda K, Uemura D. Isolation and structures of Haterumalides NA, NB, NC, ND, and NE, novel macrolides from an okinawana sponge Ircinia sp. Tetrahedron Lett 40: 6309–6312 (1999)
12. Inami M, Kawamura I, Tsujimoto S, Yasuno T, Elizabeth Lacey, Hirosumi J, Takakura S, Nishigaki F, Naoe Y, Manda T, Mutoh S. FR177391, a new anti-hyperlipidemic agent from *Serratia*. II. Pharmacological activity of FR177391. J Antibiot 58: 640–647 (2005)
13. Strobel G, Li JY, Sugawara F, Koshino H, Harper J, Hess WM. Oocydin A, a chlorinated macrocyclic lactone with potent anti-oomycete activity from *Serratia marcescens*. Microbiology 145: 3557–3564 (1999)