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

III. Microbial Conversion of FR177391 and Synthesis of FR177391 Derivatives for Its Target Protein Screening by Chemical Genetic Approaches

Motoo Kobayashi[†], Kentaro Sato[†], Seiji Yoshimura[†], Makiko Yamaoka, Shigehiro Takase[†], Mitsuru Ohkubo[†], Takashi Fujii[†], Hidenori Nakajima[†]

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Abstract FR177391 produced by *Serratia liquefaciens* No. 1821 enhances differentiation of mouse 3T3-L1 fibroblasts to adipocytes and reduces the circulating levels of triglyceride in C57BL/KsJ-db/bd mice, an obese non-insulin-dependent diabetes mellitus animal model, although its mechanism of actions remained to be unknown. Its active derivative, 20-hydroxy FR177391, and its inactive derivative, 3-hydroxy FR177391 were produced by microbial conversion of FR177391, and biotin-labeled FR177391 was synthesized from 20-hydroxy FR177391 as an active affinity ligand to identify target molecules of FR177391 by chemical genetic approaches.

Keywords FR177391, bioconversion, affinity ligand, chemical genetic approach

Introduction

FR177391 (1), produced by *Serratia liquefaciens* No. 1821, enhances differentiation of mouse 3T3-L1 fibroblasts to adipocytes and reduces the circulating levels of triglyceride in C57BL/KsJ-db/bd mice [1, 2], an obese non-insulin-dependent diabetes mellitus animal model, although its mechanism of actions remained to be unknown. Thus, identification and validation of its target protein certainly enables us to understand new biological linkage between the target and lipid metabolism.

To identify the target molecules of pharmacologically active compound, affinity purification is a well-established technique as a powerful method of ligand binding proteins. Handa *et al.* reported that receptor purification of a small molecules such as E-3330, FK506 and FR225659 using the latex beads which consist of polystyrene and polyglycidyl methacrylate copolymer [3, 4]. Schreiber *et al.* reported on the purification of histone deacetylase as trapoxin's cellular target using a trapoxin-based affinity reagent [5]. We need bioactive derivatives to prepare the ligand for the aim of target molecule identification of **1**, but it is difficult to synthesize them chemically for their low stability as natural product. Hence we tried microbial conversion which can serve as a tool to effect useful derivation reactions with cellular enzyme equipment.

In this work, we carried out microbial conversion of **1** and found its active derivative, 20-hydroxy FR177391 (**2**), and its inactive derivative, 3-hydroxy FR177391 (**3**), and synthesized biotin-labeled FR177391 (**8**) from **2** as an active affinity ligand to identify target molecules of **1**.

M. Kobayashi (Correspondng authors), **K. Sato, S. Yoshimura, M. Yamaoka, S. Takase, M. Ohkubo, T. Fujii, H. Nakajima:** Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-3, 5-chome, Tokodai, Tsukuba, Ibaraki 300-2698, Japan, E-mail: motoo.kobayashi@jp.astellas.com
[†] Present address: Astellas Pharma. Inc., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan

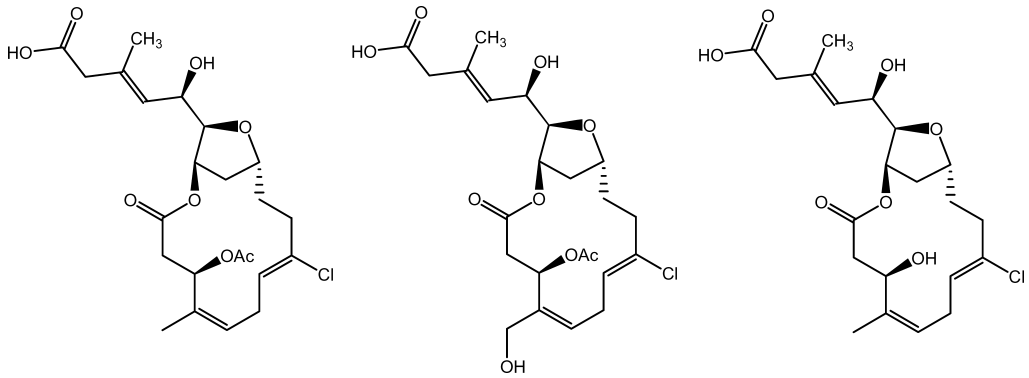


Fig. 1 Structures of FR177391 and its bioconversion products (**1**) FR177391, (**2**) 20-hydroxy FR177391, (**3**) 3-hydroxy FR177391

Materials and Methods

Chemicals and Microorganisms

FR177391 was isolated from a cultured broth of *Serratia liquefaciens* No. 1821, a strain in the Fujisawa culture collection. Strains (80 actinomycetes) were purchased from the American Type Culture Collection (ATCC), The Institute for Fermentation, Osaka (IFO), NCIMB Japan Co., Ltd. and Japan Collection of Microorganisms (JCM).

Composition of the Liquid Seed Media and Main Media

Seed Medium: AP43: sucrose 0.5%, glucose 0.5%, oatmeal 0.5%, yeast extract 0.2%, peptone 0.5%, peanut powder 0.5%, "HUMAS" (Aiaisi kabushikikaisha, Osaka, Japan) as humic acid 0.01%, polyoxyethylene sorbitan monooleate (Tween 80) 0.1% and $CaCO_3$ 0.2% (pH 7.0 adjusted with 6 N NaOH).

Main Media: AM78: corn flour 1.0%, modified starch 6.0%, pharmamedia 1.2%, dried yeast 0.8%, KH_2PO_4 0.3% and $MgSO_4 \cdot 7H_2O$ 0.3% and $FeSO_4 \cdot 7H_2O$ 0.02% (pH 6.5 adjusted with 6 N NaOH). AM86: glucose 0.5%, modified starch 2.5%, soybean flour 1.0%, dried yeast 0.5%, L-asparagine 0.2%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.05%, NaCl 0.05% and $CaCO_3$ 0.2% (pH 6.5 adjusted with 6 N NaOH).

Methods for Screening of Bioconversion Strains

Stock cultures were prepared and maintained on agar slant, and inoculated into a main media (see above). The inoculated vegetative medium (8 ml) was shaken on a reciprocating shaker (220 rpm, 5.1 cm throw) in a test tube (i.d. 18 mm×198 mm) at 30°C. After 72 hours, 500 μl of culture broth was transferred to a test tube (i.d. 13 mm× 100 mm), and FR177391 dissolved in methanol was added to a final concentration of 500 μg/ml to it. Cultures (reactions) were harvested after 16 hours of incubation. Five hundred micro liters of acetone were added to 500 μl of the main culture, shaken and centrifuged at 3000 rpm for 10 minutes. Ten micro liters of whole broth extracts were chromatographed on HPLC with 40% aqueous CH_3CN containing 0.1% TFA on a Mightysil RP-18 GP 250-4.6 (250 mm L.×4.6 mm I.D., Kanto Chemical Co., Inc., Japan) at 30°C. Biotransformation products were detected at 210 nm (UV).

LC-MS

Electro spray ionization mass spectrometry (ESI-MS) was performed on an Agilent 1100 mass spectrometer. The electrostatic-spray ion source was operated at 3.5 kV and atmosphere-vacuum transfer capillary was heated at 300°C. Full scan mass spectra were recorded from mass to charge ratio (*m*/*z*) 100 to 1300 in 1.51 seconds for the MS analysis of FR177391. The conditions for LC-MS were set up as followed: Column: Develosil 30, (3 mm), gradient system: H_2O/CH_3CN 5～100% in 10 minutes, hold, column temperature: 30°C. UV-detection at 210 nm.

Culture Conditions and Reaction Conditions for Isolation of 2

Stock culture of *Amycolatopsis azurea* JCM3275 were prepared and maintained on agar slant, and inoculated into a seed medium (AP43, see above). The inoculated vegetative medium (60 ml) was shaken on a rotary shaker (220 rpm, 5.1 cm throw) in a 225 ml Erlenmeyer flask at 30°C for about 72 hours. And 160 μl of the seed culture was inoculated to 8 ml of the sterile main media (AM78, see above) in the 16 test tubes (i.d. 18 mm×198 mm). These test tubes were shaken on a reciprocating shaker (220 rpm, 5.1 cm throw) at 30°C. After 72 hours, **1** dissolved in methanol was added to a final concentration of 500 μg/ml to the main culture. Cultures (reactions) were

harvested after 25 hours of incubation.

Isolation of 2

The reaction mixture (48 ml) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature. The mixture was filtered with an aid of diatomaceous earth and concentrated under reduced pressure to remove acetone. After addition of formic acid (final 0.1%), the mixture was passed through a column (20 ml) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with water containing 0.1% formic acid. The column was washed with 10% aqueous acetonitrile containing 0.1% formic acid (50 ml) and 20% aqueous acetonitrile containing 0.1% formic acid (63 ml) and eluted with 30% aqueous acetonitrile containing 0.1% formic acid (80 ml). The active fraction (30 ml) was diluted with equal volume of water and passed through a column (20 ml) of Daisogel SP-120-ODS-B packed with 15% aqueous acetonitrile containing 0.05% formic acid. The column was washed with 15% aqueous acetonitrile (50 ml) and eluted with methanol (30 ml). The active fraction was concentrated *in vacuo*, then separated by preparative HPLC using a reverse phase column, Mightysil RP-18 GP 250-20 (Kanto Chemical Co., Inc., Japan) with 30% aqueous acetonitrile containing 0.1% formic acid. The active fraction (29 ml) was diluted with equal volume of water and passed through a column (20 ml) of Daisogel SP-120-ODS-B packed with 15% aqueous acetonitrile containing 0.05% formic acid. The column was washed with 15% aqueous acetonitrile (50 ml) and eluted with methanol (35 ml). The active fraction (35 ml) was concentrated under reduced pressure to remove methanol to give 2.0 mg of **2**, $[\alpha]_D^{23}$ +37° (*c* 0.75 in CH_3OH).

Culture Conditions and Reaction Conditions for Isolation of 3

Stock culture of *Amycolatopsis azurea* JCM3275 were prepared and maintained on agar slant, and inoculated into a seed medium (AP43, see above). The inoculated vegetative medium (30 ml) was shaken on a rotary shaker (220 rpm, 5.1 cm throw) in a 225 ml Erlenmeyer flask at 30°C for about 72 hours. And 0.6 ml of the seed culture was inoculated to 30 ml of the sterile main media (AM86, see above) in three 225 ml Erlenmeyer flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 30°C. After 72 hours, **1** dissolved in methanol was added to a final concentration of 500 μg/liter to the main culture. Cultures (reactions) were harvested after 23 hours of incubation.

Isolation of 3

The reaction mixture (80 ml) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature. The mixture was filtered with an aid of diatomaceous earth and concentrated under reduced pressure to remove acetone. After adjusting to pH 3.0 with 6 N HCl, this mixture was extracted with 200 ml of ethyl acetate. The solvent extract (upper layer) was concentrated under reduced pressure to an oily residue. This residue was dissolved in 200 ml of water and, after addition of 0.2 ml trifluoroacetic acid (TFA), passed through a column (20 ml) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with water containing 0.1% TFA. The column was washed with 30% aqueous acetonitrile containing 0.1% TFA (42 ml) and eluted with 38% aqueous acetonitrile containing 0.1% TFA (76 ml). The active fraction (11 ml) was diluted with water to 40 ml and passed through a column (20 ml) of Daisogel SP-120-ODS-B packed with 10% aqueous acetonitrile containing 0.025% TFA. The column was washed with water (60 ml) and eluted with 85% aqueous acetonitrile (10 ml). Active fraction (10 ml) was concentrated under reduced pressure to remove acetonitrile and lyophilized to give 26 mg of **3**.

^{1}H NMR Spectroscopy

^{1}H NMR was measured on a Bruker DRX500 NMR spectrometer. Standard pulse sequences were employed for COSY.

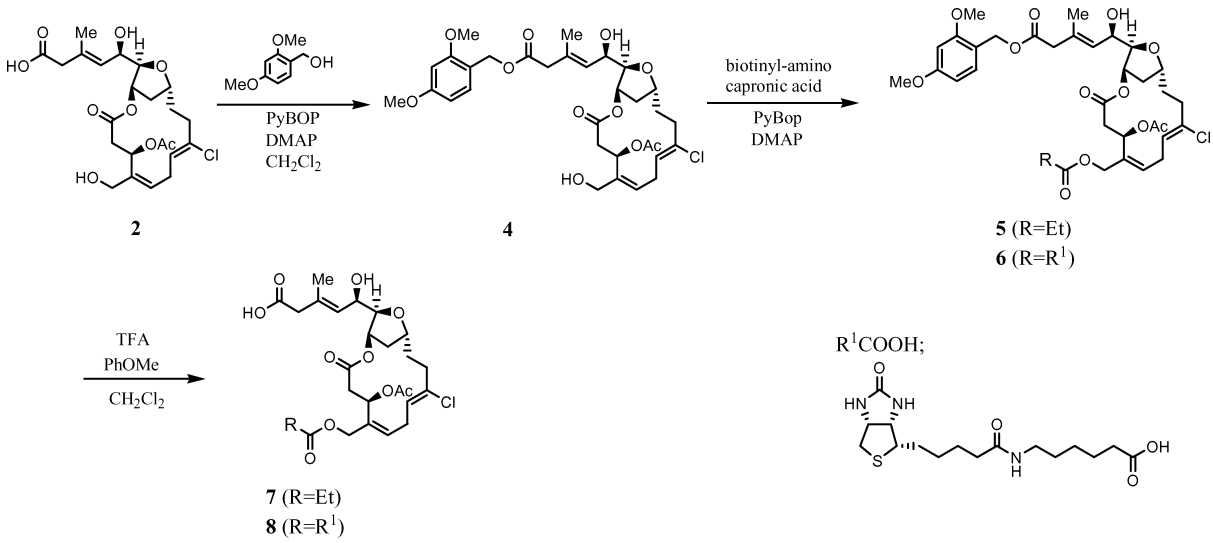
Biological Assay

Assay procedures for differentiation of mouse 3T3-L1 fibroblasts to adipocytes were conducted according to the methods described by Sato *et al*.[1].

Synthesis of 4

To an ice-cooled solution of **2** (50 mg, 103 μmol), 2,4-dimethoxybenzyl alcohol (86 mg, 513 μmol) and 4-dimethylaminopyridine (28 mg, 226 μmol) in dichloromethane (0.6 ml) was added PyBOP (64 mg, 123 μmol) and stirred 5 hours at room temperature. The resulting solution was diluted with chloroform, washed with water and brine, dried over magnesium sulfate and evaporated. The residue was purified with silica-gel column chromatography (hexane - ethyl acetate 1:1) to give target **4** (52 mg, 80%) as clear oil.

^{1}H-NMR (300 MHz, $CDCl_3$); δ 7.23 (d, 1H, J=11 Hz), 6.45 (m, 1H), 6.07 (m, 1H), 5.82 (dd, 1H, J=4.8, 11.5 Hz), 5.42 (d, 1H, J=7.5 Hz), 5.23 (m, 1H), 5.08 (s, 2H), 4.59 (dd, 1H, J=7.5, 7.5 Hz), 4.37 (d, 1H, J=14.6 Hz), 4.18 (d, 1H, J=14.6 Hz), 3.93 (m, 1H), 3.80 (s, 6H), 3.58 (m, 1H), 3.04 (d, 1H, J=14.3 Hz), 2.99 (d, 1H, J=14.3 Hz), 2.46~



Scheme 1.

2.90 (m, 6H), 2.40 (m, 1H), 2.19 (dd, 1H, J=12.1, 12.2 Hz), 2.04 (s, 3H), 1.82 (s, 3H), 1.52 (m, 1H), 1.38 (m, 1H).

Synthesis of 5

To an ice-cooled solution of **4** (1 mg, 1.6 μmol), propionic acid (0.13 mg, 1.8 μmol) and 4-dimethylaminopyridine (0.4 mg, 3.4 μmol) in dichloromethane (0.5 ml) was added PyBOP (1 mg, 1.9 μmol) and stirred 12 hours at room temperature. The solution was diluted with ethyl acetate, and the organic phase was washed with 0.5 N-HClaq, H_2O, sat.$NaHCO_3$aq and brine, and evaporated. The residue was purified on preparative TLC (ethyl acetate) to give **5** (0.5 mg, 46%).

^{1}H-NMR (500 MHz, $CDCl_3$); δ 7.23 (d, 1H, J=8 Hz), 6.46 (2H, m), 6.04 (dd, 1H, J=10, 7 Hz), 5.82 (dd, 1H, J=11, 5 Hz), 5.42 (br d, 1H, J=8 Hz), 5.31 (m, 1H), 5.20 (m, 1H), 5.09 (s, 2H), 4.93 (d, 1H, J=15 Hz), 4.74 (d, 1H, J=15 Hz), 3.94 (m, 2H), 3.81 (s, 6H), 3.09 (s, 2H), 2.93 (m, 2H), 2.84 (t, 1H, J=12 Hz), 2.66 (m, 1H), 2.52 (m, 1H), 2.38 (q, 2H, J=7 Hz), 2.20 (m, 1H), 2.10 (m, 1H), 2.04 (s, 3H), 1.84 (s, 3H), 1.53 (m, 1H), 1.44 (m, 1H), 1.17 (t, 3H, J=7 Hz).

Synthesis of 6

To an ice-cooled solution of **4** (52 mg, 82 μmol), 7-(biotinylamino)capronic acid (44 mg, 122 μmol) and 4-dimethylaminopyridine (20 mg, 163 μmol) in dichloromethane (0.7 ml) was added PyBOP (64 mg, 122 μmol) and stirred 12 hours at room temperature. The solution was evaporated and the residue was purified with silica-gel column chromatography (chloroform - methanol 1～10%) to give target **6** as slightly yellow oil.

^{1}H-NMR (300 MHz, $CDCl_3$); δ 7.49 (m, 1H), 7.42 (m, 1H), 7.22 (d, 1H, J=11 Hz), 6.47 (m, 1H), 6.06 (m, 1H), 5.84 (m, 1H), 5.42 (m, 1H), 5.32 (s, 1H), 5.22 (m, 1H), 5.10 (s, 2H), 4.93 (d, 1H, J=11.7 Hz), 4.74 (d, 1H, J=11.7 Hz), 4.53 (dd, 1H, J=6.5, 6.4 Hz), 4.51 (m, 1H), 4.32 (m, 1H), 3.92～3.77 (m, 4H), 3.83 (s, 3H), 3.82 (s, 3H), 3.62 (m, 1H), 3.25～3.11 (m, 4H), 2.96～2.61 (m, 8H), 2.38 (t, 2H, J=7.2 Hz), 2.41～2.28 (m, 2H), 2.17 (t, 2H, J=7.2 Hz), 2.10～2.24 (m, 1H), 2.05 (s, 3H), 1.82 (s, 3H), 1.77～1.20 (m, 14H).

Synthesis of 7

To a solution of **5** (0.5 mg, 0.7 μmol) and anisole (0.4 mg, 4 μmol) in dichloromethane (0.3 ml) was added trifluoroacetic acid (0.3 mg, 3 μmol) at room temperature and stirred 2 hours. The solution was evaporated and the residue was purified with silica-gel column chromatography (1～10% methanol in chloroform) to give **7** (0.2 mg, 51%).

^{1}H-NMR (500 MHz, $CDCl_3$); δ 6.04 (dd, 1H, J=11, 7 Hz), 5.82 (dd, 1H, J=11, 5 Hz), 5.56 (m, 1H), 5.32 (br s, 1H), 5.21 (br d, 1H, J=7 Hz), 4.93 (d, 1H, J=14 Hz), 4.74 (d, 1H, J=14 Hz), 4.58 (m, 1H), 4.06 (m, 1H), 3.93 (m, 1H), 3.58 (m, 1H), 3.2～3.0 (m, 2H), 2.84 (t, 1H, J=12 Hz), 2.76 (m, 1H), 2.67 (m, 1H), 2.54 (m, 1H), 2.40 (m, 1H), 2.38 (q 2H, J=7 Hz), 2.19 (m, 1H), 2.10 (m, 1H), 2.08 (s, 3H), 1.86 (br s, 3H), 1.18 (t, 3H, J=7 Hz).

Synthesis of 8

To a solution of **6** (27 mg, 28 μmol) and anisole (30 mg, 276 μmol) in dichloromethane (1 ml) was added trifluoroacetic acid (31 mg, 276 μmol) at room temperature and stirred 2 hours. The solution was evaporated and the

Table 1 Strains converting FR177391

Compound	Relative retention*	Amount of derivatives in %**	Actinomycete strain	Medium
20-Hydroxy FR177391 (**2**)	3.14	12.7	*Amycolatopsis azurea* JCM3275	AM78
3-Hydroxy FR177391 (**3**)	3.59	16.6	*Streptomyces galilaeus* ATCC31649	AM78
		26.5	*Saccharothrix aerocolonigenes* ATCC39243	AM78
		30.7	*Streptomyces capreolus* ATCC31963	AM78
		5.8	*Streptomyces remosus* JCM4073	AM78
		39.6	*Streptomyces roseochromogenes* NCBI10984	AM78
		27.6	*Actinoplanes utahensis* IFO13244	AM78
		37.3	*Nonomuraea reseoviolacea* JCM3145	AM78
		65.0	*Amycolatopsis azurea* JCM3275	AM86
		16.2	*Amycolatopsis azurea* JCM3275	AM78
FR177391 (**1**)	10			

* Relative retention (HPLC)=Rt (derivative) / Rt (FR177391)×10

** Amount of derivative in % determined by HPLC=AUC (derivative)×100% / AUC (FR177391 in compound control)

AUC: Area under curve.

residue was purified with silica-gel column chromatography (chloroform - methanol 1～15%) to give **8** as slightly clear oil.

^{1}H-NMR (500 MHz, $CDCl_3$); δ 6.09 (dd, 1H, J=3.2, 4.1 Hz), 5.48 (d, 1H, J=5.5 Hz), 5.36 (s, 1H), 5.34 (m, 1H), 5.32 (s, 2H), 4.95 (d, 1H, J=8.7 Hz), 4.72 (d, 1H, J=8.7 Hz), 4.53 (dd, 1H, J=5.2, 5.2 Hz), 4.48 (t, 1H, J=4.5 Hz), 4.48 (m, 1H), 4.30 (m, 1H), 3.97～3.87 (m, 2H), 3.52 (m, 1H), 3.25～3.11 (m, 4H), 2.96～2.79 (m, 5H), 2.71～2.56 (m, 3H), 2.45 (m, 1H), 2.37 (t, 2H, J=5.2 Hz), 2.45～2.28 (m, 2H), 2.20 (t, 2H, J=5.4 Hz), 2.10～2.04 (m, 1H), 2.04 (s, 3H), 1.80 (s, 3H), 1.79～1.28 (m, 14H).

Results and Discussion

We tested (screened) 80 actinomycetes strains, known for their ability to produce various second metabolites and transform various compounds, to convert **1** by microbial conversion and found *Amycolatopsis azurea* JCM3275 [6] hydroxylate 4-Me to give **2** and *Amycolatopsis azurea* JCM3275 [5], *Streptomyces galiaeus* ATCC31649 [7], *Saccharothrix aerocolonigenes* ATCC39243 [8], *Streptomyces capreolus* ATCC31963 [9], *Streptomyces remosus* JCM4073 [10], *Streptomyces roseochromogenes* NCIMB10984 [11], *Actinoplanes utahensis* IFO13244 [12] and *Nonomuraea reseoviolacea* JCM3145 [13] deacetylate to yield **3** (Table 1). We tested whether the cell extract of 3T3-L1 fibroblasts can convert **1** to **2** or **3**, but **1** was not converted.

Table 2 Adipogenesis-enhancing effect of FR177391 and its derivatives on 3T3-L1 fibroblasts

	1 (FR177391)	**2**	**3**	**7**
MEC* (μM)	0.1	3	>100	3

* MEC=Minimal effective concentration

The structure of **1** was inferred from observation of its pseudomolecular ion at 409 (M+H) in ESI-MS, newly generated proton signals at 4.32 ppm (2H, AB) coupled with an olefinic proton at 6.03 ppm in COSY and disappearance of the proton methyl signals at 1.88 ppm of **1** in ^{1}H NMR. The positive molecular ion of **2** at 429 (M+H) was suggestive of its deacetylated structure from **1**, which was supported by the up-field shift of 3-H in the ^{1}H NMR from 5.86 ppm of **1** to 4.63 ppm of **2**.

Then, we tested adipogenesis-enhancing effect of **1**, **2** and **3** on 3T3-L1 fibroblasts (Table 2). The compound **2** retains moderate activity while **3** completely loses the activity even in a 1000-fold concentration as MEC of **1**. We supposed that **1** was an active form in 3T3-L1 fibroblasts, because **1** was not converted in the cell extract. Moreover, we thought that **3** was not inactive due to its impaired cell permeability, because **3** had no activity in the cell-free ERK phosphorylation assay using 3T3-L1 fibroblasts [14]. This fact shows the acetyl group at position **3** is mandatory for

the biological activity of **1**. On the contrary, modification at 4-CH_3 does not give significant loss of the activity. Additionally, the compound **2** can bear in position **4** a linker terminated with biotin, which makes **2** useful to synthesize an affinity ligand to capture proteins that **1** binds. Amidation of the carboxylic acid of **1** resulted in a significant loss of the activity. Propylamide derivative of FR177391 was inactive (data not shown). Therefore, the carboxylic group of **1** was not appropriate to extend a linker terminated with biotin. In order to test whether such a ligand could bind the proteins responsible for the biological activities of **1**, we synthesized **7** from **1** as shown in Scheme 1 and examined its adipogenesis-enhancing effect (Table 2). In this synthesis, 2,4-dimethoxybenzyl ester [15] was a protective group of choice for the carboxylic acid, because **1** was found to be labile against saponification conditions or concentrated TFA. Omit of the protection yielded isomerization of $\Delta^{16,17}$ followed by γ-lactone formation. Esterification utilizing PyBOP-DMAP allowed **5**, and successive deprotection of the 2,4-dimethoxybenzyl group went very smoothly with **4** equivalents of TFA and **5** equivalents of anisole to give the targeted compound **7**. The anti-hyperlipidemic activities of **7** was equal to that of **2**, which proved the primary alcohol of **2** to be suitable for biotin label. Thus, we synthesized a biotin-labeled compound **8** in the similar method. In this synthesis, more concentrated TFA (10 equivalents) was needed for the deprotection step, probably because the high polarity of biotin structure weakened the acidity of TFA. As a result, we obtained in hand **8** as a ligand for affinity purification of the biological target molecules of **1**, and an inactive derivative **3** to validate them.

The studies of target protein screening by chemical genetic approaches are described in the following paper [14].

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