TMC-49A, a Novel Transcriptional Up-Regulator of Low Density Lipoprotein Receptor, Produced by *Streptomyces* sp. AS1345

YUTAKA KOGUCHI, YASUYUKI ASAI, SHIN-ICHI SUZUKI, MAKI NISHIO, NORIYUKI YANAKA[†],
KENJI OMORI[†], TETSUO OHNUKI* and SABURO KOMATSUBARA

Lead Generation Research Laboratory at Toda and [†]Lead Generation Research Laboratory at Kashima,
Tanabe Seiyaku Co., Ltd.,
2-50 Kawagishi-2-chome, Toda-shi, Saitama 335, Japan
[†]16-89 Kashima-3-chome, Yodogawa-ku, Osaka 532, Japan

(Received for publication October 14, 1997)

Microbial metabolites were screened for a transcriptional up-regulator of low density lipoprotein (LDL) receptor by a reporter assay. TMC-49A was discovered as an up-regulator obtained from the fermentation broth of *Streptomyces* sp. AS1345. The structure of TMC-49A was elucidated to be butyl *N*-phenethylcarbamate by spectroscopic analyses. This compound enhanced the synthesis of LDL receptor in human hepatoma HepG2 cells as assessed by a receptor binding assay. Taxonomy of the producing strain is also described.

Low density lipoprotein (LDL) receptor mediates the uptake of plasma LDL, a cholesterol rich lipoprotein, to supply cholesterol to cells¹⁾. When cellular demands for cholesterol are high, LDL receptor is actively synthesized to satisfy their demands via the uptake of plasma cholesterol. In contrast, when sterols accumulate within cells, the synthesis of LDL receptor is suppressed. This sterol negative feedback regulation is exerted to a large extent at the level of gene transcription by the specific transcriptional factors, SREBP-1 and SREBP-2^{2,3)}.

The inhibition of cholesterol biosynthesis depletes the cellular cholesterol and activates the transcription of LDL receptor gene, thereby lowering the plasma cholesterol level. This is a plausible mechanism of HMG-CoA reductase inhibitors used clinically for ameliorating an elevated level of plasma cholesterol⁴⁾. Thus, agents that activate directly the transcription of LDL receptor gene might be another candidate for development of drugs to treat hypercholesterolemia. As a result of screening ca. 10,000 microbial extracts, we found a novel compound named TMC-49A and two known compounds, trichostatin A⁵⁾ and herboxidiene⁶⁾, to up-regulate the transcription of LDL receptor. In the previous paper⁷⁾, we reported the biological properties of trichostatin A and herboxidiene. In this paper, we describe the taxonomy of the producing strain and isolation, structure elucidation, and biological properties

of TMC-49A.

Results

Taxonomy

The producing strain AS1345 was isolated from a soil sample collected in Kanagawa Prefecture, Japan. The cultural characteristics of the strain AS1345 are summarized in Table 1.

The substrate mycelium developed well and branched irregularly. Each spore chain, which was spiral type, had more than 30 spores per chain. The spores were cylindrical or ellipsoidal, with size of $0.4 \sim 0.6 \times 0.8 \sim 1.1 \mu\text{m}$, and their surface was spiny (Fig. 1). Fragmentation of substrate mycelium, sporangia, or motile spore was not observed.

The physiological properties and the utilization of carbon sources are summarized in Table 2. Analysis of the whole-cell hydrolyzate of the strain showed the presence of LL-diaminopimelic acid. On the basis of these morphological and chemical characteristics, strain AS1345 was assignable to the genus *Streptomyces*.

Production

Streptomyces sp. AS1345 was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of a seed medium composed of 0.5% glucose, 1.0% starch, 0.5% glycerol,

Table 1. Cultural characteristics of strain AS1345.

Media	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract - malt extract agar (ISP No. 2)	Good	Abundant, Pale blue purple (18-18-3*)	Yellowish brown (17-16-3)	None
Oatmeal agar (ISP No. 3)	Good	Abundant, Pale bluish purple (19-17-4)	Yellowish brown (17-16-4)	None
Inorganic salts - starch agar (ISP No. 4)	Good	Abundant, Pale bluish purple (19-18-3)	Yellowish brown (7-16-3)	None
Glycerol - asparagine agar (ISP No. 5)	Good	Abundant, Grayish purple (20-17-2)	Pale yellowish brown (7-18-2)	None
Peptone - yeast extract iron agar (ISP No. 6)	Poor	None	Dark yellowish brown (7-13-2)	None
Tyrosine agar (ISP No. 7)	Good	Abundant, Pale purple (20-18-2)	Yellowish brown (7-16-3)	None
BENNETT's agar	Good	Abundant, Pale purple (20-18-2)	Yellowish brown (7-16-3)	None

* Color codes from the Guide to Color Standard.

Fig. 1. Scanning electron micrograph of producing strain, *Streptomyces* sp. AS1345.

Bar represents 2 μ m.

0.5% Soytone, 0.2% yeast extract, 0.5% corn steep liquor, and 0.3% CaCO_3 , adjusted at pH 6.5 before autoclaving, and incubated for 6 days at 27 °C on a rotary shaker (220 rpm). One milliliter of the seed culture was transferred to a 500-ml Erlenmeyer flask containing 70 ml of a production medium composed of 0.5% glucose, 2.0% dextrin, 2.0% glycerol, 1.5% rape-seed meal, 0.5% corn steep liquor, 0.2% yeast extract, 0.3% CaCO_3 , and 0.2% Polypeptone, adjusted at pH 7.0 before autoclaving, and incubated for 5 days at 27°C on a rotary shaker (220 rpm).

Isolation

The culture broth (7 liters) of one hundred flasks was

Table 2. Physiological properties of strain AS1345.

Temperature range for growth	10 ~ 37°C
Optimum temperature for growth	27 ~ 30°C
Production of melanoid pigment	
ISP No. 6	+
ISP No. 7	—
Hydrolysis of starch	+
Decomposition of cellulose	—
Reduction of nitrate	+
NaCl tolerance	6%
Utilization of carbon source	
L-Arabinose	+
D-Fructose	+
D-Glucose	+
Inositol	±
D-Mannitol	—
Raffinose	+
L-Rhamnose	—
Sucrose	—
D-Xylose	+

+, Positive; ±, doubtful; —, negative.

extracted with ethylacetate and then concentrated *in vacuo*. The extract was subjected to a reverse-phase silica gel (YMC ODS A60) column chromatography, followed by gradient elution with $\text{CH}_3\text{CN} - \text{H}_2\text{O}$ from 4 : 6 to 5 : 5. The fractions containing TMC-49A were identified by the reporter assay to test the effect on the transcription of LDL receptor gene. The active fractions were concentrated and further purified by Sephadex LH-20 column chromatography with $\text{CH}_2\text{Cl}_2 - \text{MeOH}$ (1 : 1). After

concentration of the active fractions, purified TMC-49A was obtained as colorless oil (480 mg).

Physico-chemical Properties

Physico-chemical properties of TMC-49A are summarized in Table 3. The molecular formula of TMC-49A was established as $C_{13}H_{19}NO_2$ by high-resolution FAB-MS and NMR data. IR absorptions at 1700, 1530, and 1250 cm^{-1} implied the presence of a carbamate group.

Structure Elucidation

The ^{13}C NMR and ^1H NMR spectral data are summarized in Table 4. The data indicated the presence of a methyl, five methines, a ketone, and a monosubstituted benzene ring. Based on the results of 1D and 2D NMR (^1H - ^1H COSY and ^{13}C - ^1H long range COSY) (Fig. 2), the structure of TMC-49A was determined to be butyl *N*-phenethylcarbamate. High-resolution FAB-MS spectrum supported this structure (Fig. 2).

Table 3. Physico-chemical properties of TMC-49A.

Appearance	Colorless oil
BP ($^{\circ}\text{C}$)	120~122
Molecular formula	$C_{13}H_{19}NO_2$
HRFAB-MS (m/z)	
Found	222.1491 ($M+H$) ⁺
Calcd	222.1494
UV λ_{max} (MeOH) nm (ϵ)	260 (270)
IR ν_{max} (KBr) cm^{-1}	3325, 2950, 1700, 1530, 1250
Solubility	Soluble in MeOH, EtOAc, DMSO, CHCl_3 Insoluble in H_2O , Acetone

Biological Properties

The effect on the transcription of LDL receptor gene was assessed by a reporter assay using the recombinant cells that expressed luciferase activity under the control of LDL receptor promoter. The expression of luciferase

Table 4. ^1H and ^{13}C NMR data of TMC-49A in CDCl_3 .

Position	^{13}C (δ_{C})	^1H (δ_{H})
1	156.7 (s)	
2		4.65 (br)
3	42.1 (t)	3.42 (2H, m)
4	36.2 (t)	2.80 (2H, t, $J=6.99\text{ Hz}$)
5	138.8 (s)	
6, 10	128.8 (d)	7.29 (2H, m)
7, 9	128.6 (d)	7.18 (2H, m)
8	126.5 (d)	7.25 (1H, m)
11	64.7 (t)	4.05 (2H, t, $J=6.72\text{ Hz}$)
12	31.1 (t)	1.58 (2H, m)
13	19.1 (t)	1.36 (2H, m)
14	13.7 (q)	0.92 (3H, t, $J=7.39\text{ Hz}$)

Fig. 2. Structure of TMC-49A.

Table 5. Effect of TMC-49A on the expression of luciferase activity under the control of LDL receptor promoter in the transfected CHO cells and on specific binding of ^{125}I -LDL to LDL receptor in HepG2 cells.

Compound	Concentration (μM)	Luciferase activity (% of control ^a)	Specific binding (% of control ^b)
TMC-49A	50	1519	— ^c
	10	392	—
	2	158	185
	1	491	117
Lovastatin	1		

^a A control was the luciferase activity expressed in the recombinant CHO cells cultured in the absence of test compounds.

^b A control was the specific binding of ^{125}I -LDL to the LDL receptors of HepG2 cells cultured in the absence of test compounds.

^c —, Not determined.

activity in the cells was suppressed or activated by supplementation to culture medium of LDL or lovastatin, an HMG-CoA inhibitor, respectively (data not shown). As shown in Table 5, the expression of luciferase activity was enhanced by TMC-49A in a dose-dependent manner.

To investigate the effect of TMC-49A on the synthesis of LDL receptor, we carried out the receptor binding assay by using human hepatoma HepG2 cells and ^{125}I -LDL as a ligand. When the cells were cultured in the presence of $2\text{ }\mu\text{M}$ TMC-49A, the specific binding of ^{125}I -LDL was increased by 85%, taking the specific binding of cells cultured without test compounds as 100% (Table 5).

Discussion

In this study, TMC-49A was discovered as the novel transcriptional up-regulator of LDL receptor gene from the culture broth of *Streptomyces* sp. AS1345. The structure of TMC-49A was determined to be butyl *N*-phenethylcarbamate by the spectroscopic analyses. This is the first report on isolation of the compound with this structure from the nature, although the structure is simple. TMC-49A was confirmed to enhance the synthesis of LDL receptor in HepG2 cells. TMC-49A is a new chemical entity and a transcriptional activator of LDL receptor, that does not share a common structure with the previously reported activators, trichostatin A⁷⁾, herboxidiene⁷⁾, macrolide antibiotics⁸⁾, chenodeoxycholic acid⁹⁾, and *N*-acetyl-leucyl-leucyl-norleucinal¹⁰⁾.

The mechanism of the activation by TMC-49A has not been clarified, but it would differ from that of lovastatin since TMC-49A did not inhibit cholesterol biosynthesis (data not shown). Further study will address to the activation mechanism and give information on the regulation of LDL receptor synthesis.

TMC-49A was not so effective for the transcriptional up-regulation of LDL receptor as trichostatin A and herboxidiene⁷⁾. However, because of the simple structure of TMC-49A, it would be relatively easy to identify the moieties essential for the activation and to synthesize chemically its derivatives with higher potencies. TMC-49A has another advantage that it exhibited low cytotoxicity as compared to trichostatin A and herboxidiene. Chemical derivation of TMC-49A will be reported in near future.

Experimental

General

^1H and ^{13}C NMR spectra were recorded with a JEOL GSX-400 NMR spectrometer by using sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard. Mass spectrum was obtained with a JEOL JMS HX-411 spectrometer. UV and IR spectra were measured with a Shimadzu model UV-2200A spectrometer and a JASCO model 100 infrared spectrophotometer, respectively.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB¹¹⁾. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB¹²⁾. The substrate and aerial mass color were assigned by Guide to Color Standard, 1954 (Japan Color Research Institute). Morphological characteristics were observed with a scanning electron microscope (Hitachi S-4200). 2,6-Diaminopimelic acid in the whole cells was analyzed according to the method of BECKER *et al.*¹³⁾ and the method of HASEGAWA *et al.*¹⁴⁾.

Reporter Assay and LDL Binding Assay

The reporter assay was carried out by using the CHO cells, which expressed luciferase activity under the control of the promoter of LDL receptor gene, as described previously⁷⁾. Amounts of LDL receptor synthesized in human hepatoma HepG2 cells were measured by the receptor binding assay using the whole cells and ^{125}I -LDL as a ligand⁷⁾.

Acknowledgments

We thank Mr. JUN KOTERA for the LDL binding assay. We also thank Dr. TETSUYA TOSA and Dr. KEISUKE KAWASHIMA for their encouragement.

References

- 1) BROWN, M. S. & J. L. GOLDSTEIN: A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34~47, 1986
- 2) BRIGGS, M. R.; C. YOKOYAMA, X. WANG, M. S. BROWN & J. L. GOLDSTEIN: Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. *J. Biol. Chem.* 268: 14490~14496, 1993
- 3) WANG, X.; M. R. BRIGGS, X. HUA, C. YOKOYAMA, J. L. GOLDSTEIN & M. S. BROWN: Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. *J. Biol. Chem.* 268: 14497~14504, 1993

- 4) MABUCHI, H.; N. KAMON, H. FUJITA, I. MICHISHITA, M. TAKEDA, K. KAJINAMI, H. ITHO, T. WAKASUGI & R. TAKEDA: Effect of CS-514 on serum lipoprotein and apolipoprotein levels in patients with familial hypercholesterolemia. *Metabolism* 36: 475~479, 1987
- 5) TSUJI, N.; M. KOBAYASHI, K. NAGASHIMA, Y. WAKISAKA & K. KOIZUMI: A new antifungal antibiotic, trichosatain. *J. Antibiotics* 29: 1~6, 1976
- 6) WIDEMAN, M. M.; N. MAKAR, M. TRAN, B. ISAAC, N. BIEST & R. STONARD: Herboxidiene, a new herbicidal substance from *Streptomyces chromofuscus* A7847. *J. Antibiotics* 45: 914~921, 1992
- 7) KOGUCHI, Y.; M. NISHIO, J. KOTERA, K. OMORI, T. OHNUKI & S. KOMATSUBARA: Trichostatin A and herboxidiene up-regulate the gene expression of low density lipoprotein receptor. *J. Antibiotics* 50: 970~971, 1997
- 8) OGASAWARA, M.; N. NARUSE, A. YOSHIMURA, Y. HAMAGISHI & T. OKI: Elevation of low density lipoprotein-receptor mRNA concentration in human hepatoma HepG2 cells by macrolide antibiotics. *J. Antibiotics* 46: 866~868, 1993
- 9) KAWABE, Y.; T. SHIMOKAWA, A. MATSUMOTO, M. HONDA, Y. WADA, Y. YAZAKI, A. ENDO, H. ITAKURA & T. KODAMA: The molecular mechanism of the induction of low density lipoprotein receptor by chenodeoxycholic acid in cultured human cells. *Biochem. Biophys. Res. Comm.* 208: 405~411, 1995
- 10) WANG, X.; R. SATO, M. S. BROWN, X. HUA & J. L. GOLDSTEIN: SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* 77: 53~62, 1994
- 11) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16: 313~340, 1966
- 12) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. *J. Bacteriol.* 56: 107~114, 1948
- 13) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole cell hydrolysates. *Appl. Microbiol.* 12: 421~423, 1964
- 14) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic actinomycetes. *J. Gen. Appl. Microbiol.* 29: 319~322, 1983