Novel Lactone Compounds from *Mortierella verticillata* that Induce the Human Low Density Lipoprotein Receptor Gene: Fermentation, Isolation, Structural Elucidation and Biological Activities

KOEN A. DEKKER*, ROBERT J. AIELLO†, HIDEO HIRAI, TAISUKE INAGAKI, TATSUO SAKAKIBARA, YUMIKO SUZUKI, JOHN F. THOMPSON†, YUJI YAMAUCHI and NAKAO KOJIMA

Central Research Division, Pfizer Pharmaceuticals Inc., 5-2 Taketoyo, Aichi 470-23, Japan
†Central Research Division, Pfizer Inc., Eastern Point Road, Groton, CT 06340, U.S.A.

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Among methods of controlling hypercholesterolemia and hyperlipidemia is the direct stimulation of hepatic low density lipoprotein (LDL) receptors. Two novel lactone compounds, CJ-12,950 and CJ-13,357, containing an unusual oxime moiety, were isolated from a zygomycete *Mortierella verticillata*. These lactones are potent inducers of the LDL receptor gene *in vitro*, that enhanced LDL receptor expression in human hepatocytes 2-fold at 100 nM.

Elevated concentrations of cholesterol present in the low density lipoprotein (LDL) fraction have been demonstrated to be a major contributing factor in the development and progression of atherosclerosis. Cholesterol is derived both from the diet as well as through endogenous synthesis, and thus cells have to coordinate the uptake of plasma cholesterol by receptor-mediated endocytosis of LDL and its synthesis by the mevalonate pathway[1]. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase limit cellular cholesterol biosynthesis and such inhibition elicits, as one of the homeostasis mechanisms within the cholesterol biosynthesis pathway, an increase in the number of LDL receptors. This increases in the number of LDL receptors leads to a decrease in the circulating level of LDL cholesterol. It may be highly desirable if the synthesis of LDL receptors could be regulated directly at the level of gene expression. In a screening program of microbial secondary metabolites, the zygomycete *Mortierella verticillata* was found to produce two new lactones that increase the expression of the LDL receptor gene, resulting in higher LDL receptor numbers on the surface of liver cells. In this paper, we report the fermentation, isolation, structural elucidation and biological activities of these lactones.

Producing Strain

The producing strain, the zygomycete *Mortierella verticillata* ATCC 42662, was obtained from the American Type Culture Collection (Rockville, Maryland) and deposited as FERM BP-4786 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Tsukuba, Japan). The taxonomical properties of this strain have been reported by O'DONNELL[2].

Fermentation

Mortierella verticillata ATCC 42662 was maintained on agar slants of Medium-1 composed of potato dextrose broth (Difco) 2.4%, yeast extract 0.5% and agar 0.1%. A cell suspension from the slant was used to inoculate 50-ml tubes containing 10 ml of Medium-1. The tubes were incubated at 26°C on a shaker at 250 rpm for 7 days. The first seed culture (2 ml each) was transferred to five 500-ml flasks containing 100 ml of Medium-1. The flasks were incubated at 26°C for 4 days and used to inoculate 5 ml into eighty 500-ml flasks containing 100 ml of Medium-2 (glucose 1%, glycerol 3%, peptone 0.5% and NaCl 0.2%, pH 6.0) and 20 g of wheat bran. Incubation was carried out at 26°C for 7 days.

Isolation of Lactone Compounds

To each of the eighty 500-ml flasks, 100 ml of ethanol were added. After the combined broth was filtered, the filtrate was diluted to 20% ethanol and applied to a Diaion HP-20 (Mitsubishi Kasei) column (1 liter). After washing the column with 20% acetone, the compounds were eluted with 60% acetone. After the evaporation of acetone, the oily residue was loaded on a silica gel column (300 ml, Merck Kieselgel 60, 70~230 mesh). The column was washed with *n*-hexane and fractions showing activity were recovered by eluting with 1.5 liters of ethyl acetate-methanol (3:1). The fractions were further applied to an ODS column (Shiseido, Capcell Pak C18 SG-120, 30×250 mm) and fractions showing activity were recovered by eluting with acetonitrile-water (22:78). The fraction was applied to a Hibar-NH2 column (Merck, 10×250 mm) and two lactones showing activity were eluted with ethyl acetate-methanol (5:1), CJ-12,950 (**1**, 89 mg) and CJ-13,357 (**2**, 4.9 mg) (Fig. 1).

Physico-chemical Properties

The physico-chemical properties of CJ-12,950 (**1**) and CJ-13,357 (**2**) are summarized in Table 1. The compounds were obtained as white powder and are soluble in methanol, ethyl acetate, chloroform and acetone, but insoluble in water and *n*-hexane. The UV spectra of **1** and **2** were similar to each other, suggesting the presence of a similar chromophore. The IR absorption bands at 3285 cm^{-1} of **1** and 3405 cm^{-1} of **2** implied the presence of hydroxy group and the bands at 1701 cm^{-1} of **1** and 1703 cm^{-1} of **2** implied carboxylic ester, respectively.

Structure Elucidation

Structure Elucidation of CJ-12,950 (1)

The ^{1}H and ^{13}C NMR spectra (DMSO-d_6) exhibited 26 proton and 23 carbon signals, respectively, as summarized in Table 2. The DEPT spectra indicated the presence of one $-CH_3$, two $-CH_2-$, five $-CH-O-$, ten $=CH-$, three $-C=$ and two carbonyls. The ^{13}C-^{1}H COSY experiment established the connectivity of the proton and

Fig. 1. Structures of CJ-12,950 (**1**) and CJ-13,357 (**2**).

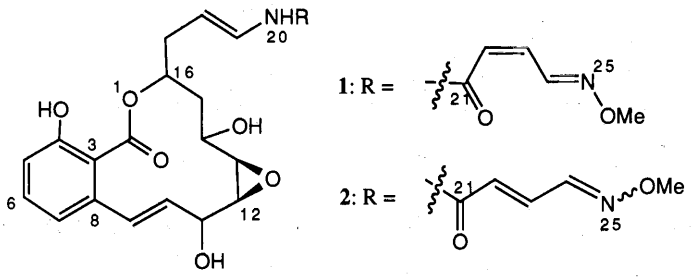


Table 1. Physico-chemical properties of CJ-12,950 (**1**) and CJ-13,357 (**2**).

	CJ-12,950 (**1**)	CJ-13,357 (**2**)
Appearance	White powder	White powder
Formula	$C_{23}H_{26}N_2O_8$	$C_{23}H_{26}N_2O_8$
HRFAB-MS	$(M-H)^-$	$(M+H)^+$
Calcd for $C_{23}H_{25}N_2O_8$:	457.1611	459.1767
Found:	457.1613	459.1783
$[\alpha]_D$ (25℃, MeOH)	+99.5° (*c* 0.22)	+107.8° (*c* 0.23)
UV λ_{max} (MeOH) nm (ε)	213.0 (39,000)	210.8 (58,000)
	259.0 (24,000)	256.2 (35,000)
	284.0 (25,000)	283.4 (37,000)
IR ν_{max} (KBr) cm^{-1}	3285, 1701, 1652	3405, 1703, 1655
TLC (Rf)		
CH_2Cl_2/CH_3OH = 5:1[a]	0.63	0.64
Visualized color[b]	Brown	Brown

[a]Nagel Sil G-25.
[b]Sprayed with 1% (w/w) vanillin/conc. H_2SO_4, then heated (~120℃).

Table 2. ^{1}H and ^{13}C NMR assignments of CJ-12,950 (**1**) in DMSO-d_6.

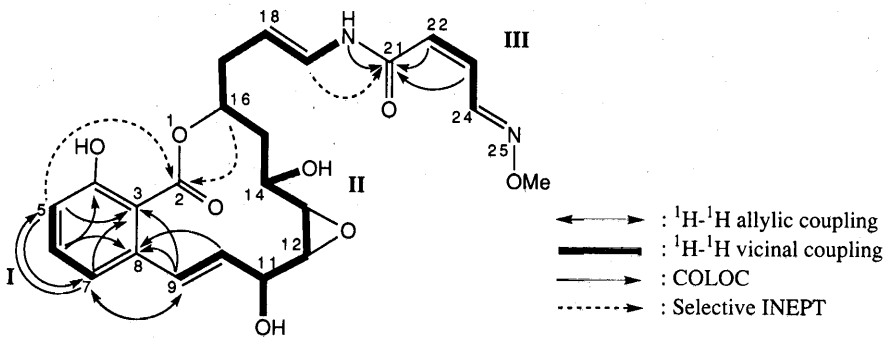
Position	^{13}C		^{1}H
2	166.9	s	
3	120.2	s	
4	154.9	s	
5	114.8	d	6.81 (d, J = 8.3 Hz)
6	130.0	d	7.19 (dd, J = 7.3, 8.3 Hz)
7	119.5	d	6.68 (d, J = 7.3 Hz)
8	135.8	s	
9	131.1	d	6.50 (d, J = 16.4 Hz)
10	133.6	d	5.69 (dd, J = 8.8, 16.4 Hz)
11	69.5	d	4.48 (d, J = 8.8 Hz)
12	58.5	d	3.06 (d, J = 4.2 Hz)
13	58.3	d	2.75 (dd, J = 4.2, 8.6 Hz)
14	59.8	d	3.97 (m)
15	40.3	t	1.81 (2H, m)
16	71.5	d	5.16 (m)
17	34.9	t	2.35 (2H, m)
18	107.8	d	5.21 (dt, J = 7.4, 14.4 Hz)
19	125.5	d	6.73 (dd, J = 10.0, 14.4 Hz)
20			10.25 (d, J = 10.0 Hz)
21	161.6	s	
22	126.3	d	6.11 (dd, J = 0.7, 11.5 Hz)
23	133.3	d	6.52 (dd, J = 10.3, 11.5 Hz)
24	147.5	d	8.99 (dd, J = 0.7, 10.3 Hz)
25-OMe	61.9	q	3.86 (s)
4-OH			9.90 (brs)
11-OH			5.46 (brs)
14-OH			5.05 (brs)

Internal reference: DMSO-d_6, δ 39.5 for ^{13}C and δ 2.50 for ^{1}H.

carbon atoms (Table 2). The HRFAB-MS gave a parent ion peak at m/z 457.1613 [$(M-H)^-$; calcd for $C_{23}H_{25}N_2O_8$, 457.1611]. These results together with ^{13}C NMR spectroscopic analysis proposed the molecular formula $C_{23}H_{26}N_2O_8$ for **1**. The degree of unsaturation from molecular formula was 12: seven were assigned to double bonds including one imine (thirteen sp^2 carbons at δ 107.8, 114.8, 119.5, 120.2, 125.5, 126.3, 130.0, 131.1, 133.3, 133.6, 135.8, 147.5 and 154.9), two to carbonyl groups (δ 161.6 and 166.9) and thus the remainder must be to the three rings of **1**.

Extensive connectivity was established through interpretation of ^{1}H-^{1}H COSY, COLOC (correlation spectroscopy *via* long range coupling) and Selective INEPT[3] spectra. ^{1}H-^{1}H COSY showed the three partial structures: I [–CH=CH–CH=]; II [–CH=CH–CH(–OH)–CH(–O–)–CH(–O–)–CH(–OH)–CH_2–CH(–O–)–CH_2–CH=CH–NH–] and III [–CH=CH–CH=] as shown in Fig. 2. In the partial structure I, the long range couplings were observed from H-5 (δ 6.81) to C-3 (δ 120.2) and C-7 (δ 119.5), from H-6 (δ 7.19) to C-4 (δ 154.9) and C-8 (δ 135.8) and from H-7 (δ 6.68) to C-3 and C-5 (δ 114.8) in COLOC. The partial structure I therefore extended to be 1,2,3-trisubstituted benzene. An allylic coupling between H-7 and H-9 (δ 6.50) in ^{1}H-^{1}H COSY indicated the attachment of C-9 to C-8. This connection was supported by the long range couplings from H-9 to C-3 and C-8 and from H-10 (δ 5.69) to C-8 in COLOC. The chemical shifts of the benzene carbons (C-3 ~ C-8) and the carbonyl carbon C-2 (δ 166.9) indicated the 6-substituted salicylic ester. The NOE observation between the phenolic proton 4-OH (δ 9.90) and H-5 confirmed the position of the hydroxy group. The long range coupling from H-16 (δ 5.16) and H-5 to the carbonyl carbon C-2 in Selective INEPT revealed the 12-membered lactone. The presence of two hydroxy groups at C-11 and C-14 were confirmed by the cross peaks between the oxy-methine proton and the hydroxy proton, H-11 (δ 4.48)/OH (δ 5.46) and H-14 (δ 3.97)/OH (δ 5.05) in ^{1}H-^{1}H COSY, respectively. The presence of

Fig. 2. ^1H-^1H COSY, COLOC and Selective INEPT experiments of **1**.



the epoxide at C-12 and C-13 was confirmed by the C–H coupling constants at C-12 (J_{C-H} 175.1 Hz) and C-13 (J_{C-H} 173.1 Hz), which were obtained by nondecoupled-INEPT, and the high field chemical shifts of C-12 (δ_H 3.06, δ_C 58.6) and C-13 (δ_H 2.75, δ_C 58.3). The coupling constant between H-12 and H-13 (4.2 Hz) showed the *cis* configuration of the epoxide. Two double bonds at C-9 and C-18 were determined to be *trans* by the coupling constants (J_{H9-H10} 16.4 Hz, $J_{H18-H19}$ 14.4 Hz).

Two partial structures II and III were connected by COLOC and Selective INEPT. The vicinal coupling between H-19 (δ 6.73) and a doublet NH proton (δ 10.25) in ^1H-^1H COSY and the cross peaks from NH proton to C-21 (δ 161.6) in COLOC indicated the enamide structure. This partial structure was further supported by the long range coupling from H-19 to C-21 in Selective INEPT. In addition, long range couplings from H-22 (δ 6.11) and H-23 (δ 6.52) to C-21 in COLOC indicated the connection of C-22 to carbonyl carbon C-21. The geometry of the double bond at C-22 was determined to be *cis* by the coupling constant ($J_{H22-H23}$ 11.5 Hz).

Finally, the remaining -N and -OMe should be attached to C-24 to form the oxime based on the comparable ^1H and ^{13}C chemical shifts with that of 1-oxo-(2*Z*)-penten-4-one *anti*-1-*O*-methyloxime (**3**), which was synthesized by modification of the Mackay procedure[4]. As shown in Fig. 3, the ^1H and ^{13}C chemical shifts (CD_3OD) of the imine carbon C-24 (δ_H 8.96, δ_C 148.7) and the olefin carbons at C-23 (δ_H 6.48, δ_C 135.5) and C-22 (δ_H 6.05, δ_C 126.2) for **1** were very similar to C-1 (δ_H 8.72, δ_C 149.4), C-2 (δ_H 6.46, δ_C 136.0) and C-3 (δ_H 6.46, δ_C 131.4) for **3**, respectively. The *anti* geometry of the oxime was also proved by NOE observation between H-24 and methoxy proton (δ 3.89). The structure of **1** was thus elucidated as shown in Fig. 1, although the stereochemistry at C-11, C-14 and C-16 remains to be established.

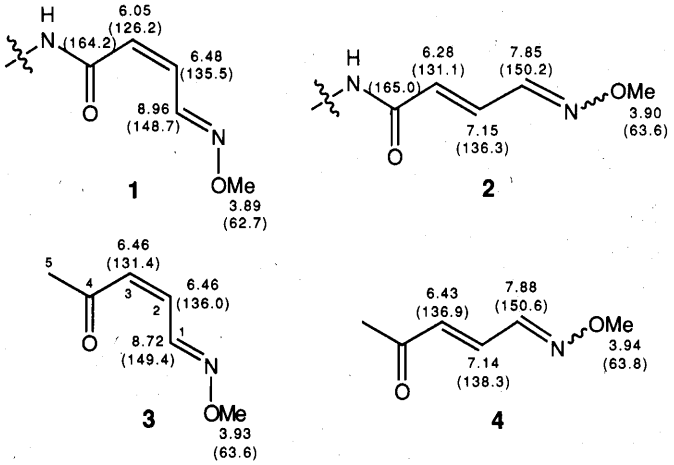
Structure Elucidation of CJ-13,357 (**2**)

The spectral data of **2** obtained from HRFAB-MS [*m/z* 459.1783, $(M+H)^+$, calcd for $C_{23}H_{27}N_2O_8$, 459.1767] and NMR experiments suggested its structure as a *trans* olefin isomer at C-22 of **1**. The ^{13}C NMR spectra (CD_3OD) of **2** were similar to those of **1** except for three signals of the olefin carbons of C-22 (δ 131.1) and C-23 (δ 136.3), and the imine carbon C-24 (δ 150.2) for **2** (Fig. 3). In the ^1H NMR (CD_3OD) of **2**, the coupling constant between H-22 (δ 6.28) and H-23 (δ 7.15) was 15.4 Hz, indicating that the geometry of olefin was *trans*. This was also suggested by a comparison of ^1H and ^{13}C NMR spectra between **2** and 1-oxo-2(*E*)-penten-4-one 1-*O*-methyloxime (**4**), which was obtained by isomerization of **3** with I_2[5]. The geometry of the oxime was not determined since both of NOEs between methoxy proton (δ 3.94) and H-23 (δ 7.14) and between methoxy proton and H-24 (δ 7.88) were observed. Thus the structure of **2** was determined as shown in Fig. 1.

Biological Properties

To detect compounds which enhance expression of the LDL receptor gene we made use of HepG2 cells (untransformed) transfected with a reporter construct consisting of 1,500 base pairs of the proximal promoter from the LDL receptor gene fused to the firefly luciferase gene (detailed construction to be described elsewhere). CJ-12,950 and CJ-13,357 showed potent luciferase reporter gene inducing activity (Fig. 4). Both compounds enhanced the expression two-fold at 0.05 μg/ml (100 nM), indicating that the stereochemistry of the oxime has little effect on the activity. These compounds showed no activity when tested at 10 μg/ml with a luciferase reporter gene construct under control of the SV40 promoter,

Fig. 3. ^{1}H and ^{13}C NMR chemical shifts of **1**~**4** in CD_3OD.



Numbers in parentheses are ^{13}C NMR chemical shifts and others are ^{1}H NMR chemical shifts.

Fig. 4. Effects of CJ-compounds on the reporter gene.

▲ CJ-12,950, ○ CJ-13,357.

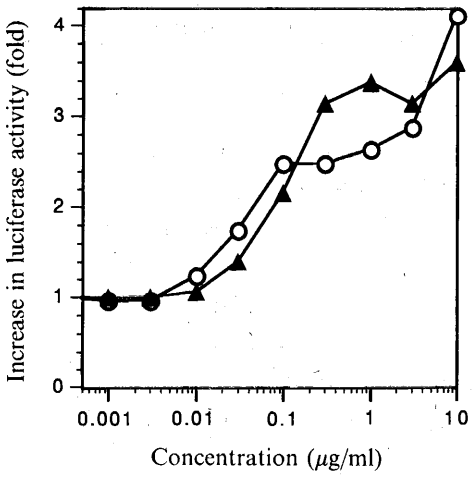
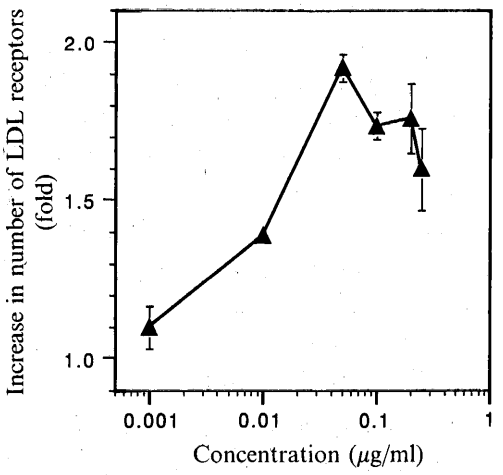


Fig. 5. Effect of CJ-12,950 on the number of LDL receptors of HepG2 cells.



suggesting specificity for the LDL receptor gene. That induction of the gene indeed resulted in an increase of the number of LDL receptors on the surface of HepG2 cells was confirmed by ELISA (Fig. 5). At the dose that enhanced expression of the reporter gene two-fold (0.05 μg/ml), the number of LDL receptors also doubled.

Discussion

Several natural products have been reported to enhance expression of the LDL receptor gene[6~9], but none of them are structurally related to CJ-12,950 or CJ-13,357, or appears to be as potent as the CJ-compounds. Furthermore, some of these reported compounds affect tubulin polymerization or lysosomal pH and probably act indirectly by interfering with cholesterol trafficking or metabolism[8~10]. We have not seen any effect of CJ-12,950 and CJ-13,357 on tubulin polymerization, lysosomal pH or cholesterol synthesis. When the changes in protein-DNA interactions induced by these compounds are examined by *in vivo* footprinting (data not shown), protein binding similar to that induced by other LDL receptor upregulators is observed[11,12], indicating a direct upregulation of the promoter.

The lactone part of the described compounds is similar

to lasiodiplodin and curvularin, and is probably formed by serial head-to-tail linkage of acetic acid units[13,14]. The oxime part appears to be unusual, and it would therefore be interesting to explore its biosynthetic pathway.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO Ubest-30; IR, Shimadzu IR-470; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; HRFAB-MS, KRATOS model IS; Optical rotations, JASCO DIP-370 with a 10 cm cell.

CJ-13,357 (2)

^{1}H NMR (CD_3OD) δ 7.85 (1H, d, J=10.3 Hz), 7.21 (1H, dd, J=8.1 and 7.6 Hz), 7.15 (1H, dd, J=15.4 and 10.3 Hz), 6.84 (1H, d, J=14.3 Hz), 6.79 (1H, d, J=8.1 Hz), 6.71 (1H, d, J=7.6 Hz), 6.62 (1H, d, J=16.3 Hz), 6.28 (1H, dd, J=15.4 and 1.1 Hz), 5.76 (1H, dd, J=16.3 and 9.0 Hz), 5.37 (1H, dt, J=14.3 and 7.3 Hz), 5.30 (1H, m), 4.59 (1H, d, J=9.0 Hz), 4.15 (1H, dt, J=8.9 and 4.0 Hz), 3.90 (3H, s), 3.19 (1H, d, J=4.1 Hz), 2.85 (1H, dd, J=8.9 and 4.0 Hz), 2.48 (2H, t, J=7.3 Hz), 1.97 (2H, m); ^{13}C NMR (CD_3OD) δ 171.0 (s), 165.0 (s), 158.0 (s), 150.2 (d), 139.2 (s), 136.3 (d), 135.1 (d), 134.7 (d), 132.7 (d), 131.1 (d), 127.3 (d), 122.0 (d), 121.4 (s), 116.9 (d), 111.6 (d), 74.4 (d), 72.6 (d), 63.6 (q), 63.0 (d), 60.8 (d), 60.0 (d), 42.1 (t), 37.3 (t).

Preparation of 1-Oxo-(2*Z*)-penten-4-one *anti*-1-*O*-Methyloxime (3)

To *O*-methylhydroxylamine hydrochloride (3.09 g, 36.9 mmol) and NaOH (1.35 g, 33.7 mmol) in water (43.2 ml) was added 1-oxo-2(*Z*)-penten-4-one[5] (3.3 g, 33.7 mmol) at room temperature. After stirring for 4 hours at room temperature, the reaction mixture was saturated with NaCl and extracted with methylene chloride. After drying over $MgSO_4$, the methylene chloride extract was filtered and concentrated. The residue was chromatographed on silica gel (Merck, Kieselgel 60, 20 g), eluted with a 9 : 1 mixture of MeOH and $CHCl_3$ to give 290 mg of a 5 : 1 mixture of **3** and 1-oxo-(2*Z*)-penten-4-one *syn*-1-*O*-methyloxime (**3'**). **3**: ^{1}H NMR (CD_3OD) δ 8.72 (1H, dd, J=7.3 and 2.2 Hz), 6.46 (2H, m), 3.93 (3H, s), 2.26 (3H, s); ^{13}C NMR (CD_3OD) δ 201.3 (s), 149.4 (d), 136.0 (d), 131.4 (d), 63.6 (q), 32.2 (q). **3'**: ^{1}H NMR (CD_3OD) δ 8.03 (1H, dd, J=9.9 and 1.1 Hz), 6.95 (1H, dd, J=10.2 and 9.5 Hz), 6.42 (1H, d, J=9.5 Hz), 3.97 (3H, s), 2.21 (3H, s); ^{13}C NMR (CD_3OD) δ 201.2 (s), 145.3 (d), 133.0 (d), 127.4 (d), 63.4 (q), 32.6 (q).

Preparation of 1-Oxo-2(*E*)-penten-4-one 1-*O*-Methyloxime (4)

To a solution of a 5 : 1 mixture of **3** and **3'** (27 mg, 0.212 mmol) in ether (1.35 ml) were added a few crystals of iodine at room temperature. After stirring for 3 hours at room temperature, the reaction mixture was washed with an aqueous sodium thiosulfate solution. After drying over $MgSO_4$, the ether extract was filtered and concentrated. The residue was chromatographed on silica gel (Merck, Kieselgel 60, 2.7 g), eluted with a 9 : 1 mixture of MeOH and $CHCl_3$ to give 25 mg of **4**. ^{1}H NMR (CD_3OD) δ 7.88 (1H, d, J=9.9 Hz), 7.14 (1H, dd, J=16.1 and 9.9 Hz), 6.43 (1H, d, J=16.1 Hz), 3.94 (3H, s), 2.31 (3H, s); ^{13}C NMR (CD_3OD) δ 201.1 (s), 150.6 (d), 138.3 (d), 136.9 (d), 63.8 (q), 28.2 (q).

Luciferase Assay and Enzyme Linked Immunosorbent Assay (ELISA)

Human hepatoma HepG2 cells (untransformed) transfected with the reporter construct (plasmid pLuxF6) were grown (37°C, 5% CO_2) in DMEM medium (Gibco) supplemented with 10% fetal calf serum and 1.6 mg/ml G418 (Gibco). The cells were transferred to 96-well plates (5×10^4 cells/well) and incubated overnight. The next day a sample was added to the well and incubation was continued overnight. The cell monolayer was washed with PBS and lysed in 100 μl buffer containing 25 mM Tris-PO_4 (pH 8.0), 16% (v/v) glycerol, 1% Triton X-100, 8 mM $MgCl_2$ and 1 mM EDTA. A 50 μl sample of the lysate was added to 100 μl lysis buffer containing 160 μM ATP and 50 μM luciferin. Luciferase activity was measured using a luminometer (Dynatech, Virginia). Antibody for the LDL receptor ELISA was obtained from Progen (Germany).

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