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A Cholesterol Biosynthesis Inhibitor from *Rhizopus oryzae*

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A bile acid derivative, methyl cholate (**1**), was isolated from EtOAc extract of the fungus *Rhizopus oryzae* as a cholesterol biosynthesis inhibitor. It showed moderate inhibitory activity on cholesterol biosynthesis in human Chang liver cells. Compound **1** exhibited inhibitory effect on the later step of cholesterol biosynthesis, indicating that its action mode is different from that of statins that act on the HMG-CoA reductase.

Key words: Cholesterol biosynthesis inhibitor, Methyl cholate, *Rhizopus oryzae*

INTRODUCTION

Higher levels of total and low-density lipoprotein (LDL) cholesterol in the blood are known to be a major risk factor of coronary heart disease. Clinical studies have indicated that lowering of the LDL cholesterol levels reduces the risk factor of coronary heart disease (Lipid Research Clinics Program, 1984a; 1984b; Shepherd *et al.*, 1995; Brown and Goldstein, 1996).

One of the most efficient approaches in the regulation of total and LDL cholesterol levels has been known to be inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, which is a rate-limiting enzyme that controls the level of mevalonate in endogenous cholesterol biosynthesis (Endo and Hasumi, 1993; Robbers *et al.*, 1996). However, HMG-CoA reductase inhibitors have been shown also to decrease the synthesis of other biologically important isoprenoid compounds derived from mevalonate, e.g. ubiquinone, dolichol, isopentenyl *t*-RNA and isoprenylated proteins. Thus, there has been continued interest in developing hypolipidemic agents that inhibit the enzymes involved specifically in the later stages of cholesterol biosynthesis (Gerst *et al.*, 1988; Biller *et al.*, 1991; Abe *et al.*, 1994).

In the previous studies, we have established a modified *in vitro* assay system to screen cholesterol biosynthesis inhibitors using Chang liver cell line (Shin *et al.*, 1999; Alam *et al.*, 2001; Kim *et al.*, 2003). The cultured human liver cell lines have been used as an effective model for screening of cholesterol biosynthesis inhibitors from natural sources including plant materials, foods and microorganisms (Grabley *et al.*, 1992; Gebhardt, 1993; Gebhardt, 1998). Moreover, these methods have been shown to provide valuable profiles for evaluation of inhibition mode against cholesterol biosynthesis based on the analysis of incorporation and distribution of radiolabeled precursor into nonsaponifiable lipids (Metherall *et al.*, 1996; Trenin *et al.*, 2003).

In our on-going search for new cholesterol biosynthesis inhibitors from microorganisms, the EtOAc extract of the fungus *Rhizopus oryzae* showed inhibitory activity in the assay system. Bioactivity-guided fractionation of the extract led to the isolation of methyl cholate (**1**), a bile acid derivative, as an inhibitor of cholesterol biosynthesis *in vitro*. This paper describes the isolation, structure elucidation and biological activity of compound **1**.

MATERIALS AND METHODS

General procedure

^{1}H-(500 MHz) and ^{13}C-(125 MHz)NMR spectra were obtained on a Varian Unity INOVA 500 spectrometer. Chemical shifts were expressed in parts per million (ppm) relative to TMS as the internal standard, and coupling constants (*J*) were given in Hertz. MS were obtained on Varian Saturn 4D mass and Jeol JMS-AX505WA spectrometers. TLC was carried out on Merck Silica gel F_{254}-precoated glass plates. Preparative HPLC was carried out on a Waters 600E multisolvent delivery system using Waters μPorasil silica HPLC columns.

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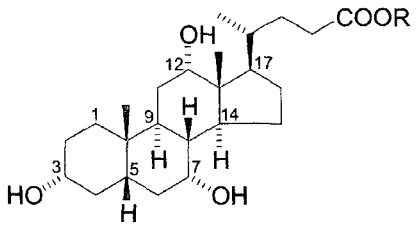

1 R= CH_3
2 R= H

Fig. 1. Structures of compounds **1** and **2**

Microorganisms and culture condition

The microorganisms were obtained from Korean Collection for Type Cultures (KCTC). The fungi *Rhizopus oryzae* (KCTC6949) and *Trichophyton mentagrophytes* (KCTC6085) were cultured at 25°C for 4 days in 1 L Erlenmeyer flasks, each containing 400mL of YM medium composed of a basal medium of dextrose (10 g/L), peptone (5 g/L), yeast extract (3 g/L) and malt extract (3 g/L).

Extraction and isolation

Total 20 L of liquid culture broth of *R. oryzae* was extracted with EtOAc (20 L × 3), and the organic layer was concentrated *in vacuo*. The EtOAc extract (1.5 g) was chromatographed on a Si gel column using a $CHCl_3$-MeOH ($CHCl_3$ 100% → 30%) gradient system to give ten fractions. Fraction 5 (377 mg) was rechromatographed on a HPLC (μPorasil, 19 × 150 mm) column using CH_2Cl_2-MeOH (85:15) to give eleven subfractions. Subfraction 9 (27.5 mg) was purified by HPLC (μPorasil, 7.8 × 300 mm) using $CHCl_3$-MeOH (98:2) to afford compound **1** (7.5 mg).

Total 16 L of culture broth of *T. mentagrophytes* was extracted with EtOAc (16 L × 3) and concentrated *in vacuo*. The EtOAc extract (1.2 g) was chromatographed on a Si gel column using a $CHCl_3$-MeOH (15:1→1:1) gradient system to give five fractions. Fraction 5 (583 mg) was rechromatographed on an MPLC column using CH_2Cl_2-MeOH-H_2O (100:12:0.2→65:35:5) to give eight subfractions. RP_{18}-MPLC of subfraction 5 (222 mg) using 65% MeOH yielded compound **2** (32.2 mg).

Methyl cholate (**1**, $C_{25}H_{42}O_5$)
Amorphous powder; EI-MS *m/z*: 423 $[M+H]^+$; ^{1}H-NMR (pyridine-d_5) δ 4.22 (brs, 1H, H-12), 4.08 (brs, 1H, H-7), 3.74 (m, 1H, H-3), 3.62 (s, 3H, H-25), 3.10 (q, *J*= 12 Hz, 1H, H-4_{ax}), 2.91 (brt, *J*= 12 Hz, 1H, H-14), 2.73 (m, 1H, H-9), 2.41 (m, 1H, H-23), 1.16 (d, *J*= 5.5 Hz, 3H, H-21), 1.00 (s, 3H, H-19), 0.79 (s, 3H, H-18); ^{13}C-NMR (pyridine-d_5) δ 174.4 (C-24), 72.4 (C-12), 71.8 (C-3), 67.7 (C-7), 51.3 (C-25), 47.1 (C-17), 46.9 (C-13), 42.7 (C-5), 42.5 (C-14), 41.0 (C-8), 40.1 (C-4), 36.3 (C-20), 35.9 (C-1), 35.9 (C-10), 35.3 (C-6), 31.7 (C-22), 31.5 (C-23), 31.2 (C-2), 29.7 (C-11), 28.1 (C-16), 27.4 (C-9), 23.8 (C-15), 23.2 (C-19), 17.5 (C-21), 13.0 (C-18).

Cholic acid (**2**, $C_{24}H_{40}O_5$) Amorphous powder; FAB-MS *m/z*: 431 $[M+Na]^+$; ^{1}H-NMR (CD_3OD) δ 3.95 (brs, 1H, H-12), 3.80 (d, *J*= 2.5 Hz, 1H, H-7), 3.37 (tt, *J*= 4.5, 11 Hz, 1H, H-3), 1.02 (d, *J*= 6.0 Hz, 3H, H-21), 0.91 (s, 3H, H-19), 0.91 (s, 3H, H-18); ^{13}C-NMR (CD_3OD) δ 178.4 (C-24), 74.2 (C-12), 73.0 (C-3), 69.2 (C-7), 48.2 (C-17), 47.6 (C-13), 43.3 (C-5), 43.1 (C-14), 41.1 (C-8), 40.6 (C-4), 36.9 (C-20), 36.6 (C-1), 36.0 (C-10), 36.0 (C-6), 32.5 (C-22), 32.2 (C-23), 31.3 (C-2), 29.7 (C-11), 28.8 (C-16), 28.0 (C-9), 24.4 (C-15), 23.3 (C-19), 17.8 (C-21), 13.1 (C-18).

Evaluation of inhibitory activity against cholesterol biosynthesis

The isolates were evaluated for their ability to inhibit cholesterol biosynthesis in Chang liver cells *in vitro*. Chang liver cells were seeded at 5×10^4 cells in 24 well plate in 0.5 mL of MEM media with 10% FBS and preincubated for 24 h. The cells were washed and cultured with serum-free media Hepatozyme-SFM (GibcoBRL) for 4 h. After pretreatment with a test compound for 2 h, ^{14}C-labeled acetate (2.5 μCi/well) was added to the culture medium and further incubated for 2 h. The cells were treated with 0.1 N NaOH and saponified with 6% KOH in MeOH (w/v), and the nonsaponifiable lipid was extracted with *n*-hexane. The *n*-hexane extract was developed on TLC with $CHCl_3$-Et_2O (19:1) solvent system, and the synthesized radioactive compounds were analyzed by a phosphor image analyzer (BAS-1500).

RESULTS AND DISCUSSION

In a search for new cholesterol biosynthesis inhibitors from microbial secondary metabolites, the EtOAc extract of *R. oryzae* showed cholesterol biosynthesis inhibitory activity in human Chang liver cells *in vitro*. Bioactivity-guided fractionation resulted in the isolation of compound **1**. EI-MS spectrum of **1** showed an $[M+H]^+$ peak at *m/z* 423. The ^{1}H-NMR spectrum of compound **1** showed signals for three hydroxymethines at δ 4.22 (H-12), 4.08 (H-7) and 3.74 (H-3), two tertiary methyls at δ 1.00 (H-19) and 0.79 (H-18), a secondary methyl at δ 1.16 (d, *J*= 5.5 Hz, H-21), and a methoxyl group at δ 3.62 (H-25). Its ^{13}C-NMR spectrum exhibited a carbonyl carbon signal at δ 174.4 which indicated this compound has a methyl ester functionality. Compound **1** was identified as methyl cholate by the comparison of its spectral data with those reported previously in the literature (Goto *et al.*, 1987; Dias *et al.*, 2000) (Fig. 1).

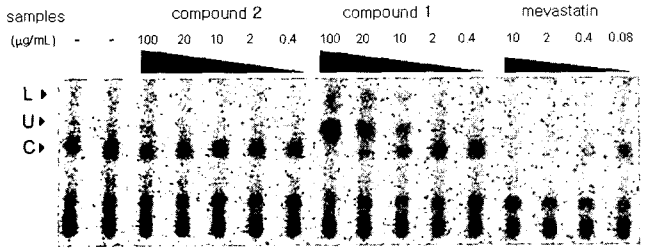

Fig. 2. The inhibitory activities of **1** and **2** on cholesterol biosynthesis in Chang liver cells. L: lanosterol, C: cholesterol, U: unidentified product

Compound **2** was obtained as colorless amorphous powder by column chromatography. Its ^{1}H- and ^{13}C-NMR spectral features were closely related to those of compound **1** except for the absence of methoxyl signal at δ 3.62. ^{13}C-NMR spectrum of **2** showed the signal of a carboxylic acid at δ 178.4. FAB-MS spectrum of **2** showed an $[M+Na]^+$ peak at *m/z* 431. Thus, structure of **1** was elucidated as cholic acid based on the spectroscopic data (Fig. 1).

Inhibitory activities of compounds **1** and **2** against cholesterol biosynthesis in Chang liver cells are shown in Fig. 2. A bile acid derivative, methyl cholate (**1**) exhibited cholesterol biosynthesis inhibition in a dose-dependent mode and moderate cholesterol biosynthesis inhibitory activity with an IC_{50} value of 13 μg/mL when compared with that of mevastatin (0.3 μg/mL), a commercially available HMG-CoA reductase inhibitor. However, compound **1** showed an inhibitory profile different from that of mevastatin. Analysis of the extracts of cells treated with **1** revealed the presence of accumulated lanosterol (L) and unidentified product (U) in the sterol fraction as evidenced by TLC, whereas no labeled intermediate was detected in case of mevastatin under the same condition. It is postulated that compound **1** may inhibit cholesterol biosynthesis at a later stage in cholesterol biosynthetic pathway. In addition, compound **1** is structurally different from compound **2** in that it contains a methoxyl group in the side chain, but compound **2** was inactive even at 100 μg/mL. It is interesting to note that the compound **2**, a bile acid closely related with **1** in structure, was inactive against cholesterol biosynthesis in human liver cells. To the best of our knowledge, this is the first report on inhibitory effect of methyl cholate (**1**) against cholesterol biosynthesis.

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