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Isolation and Structure Determination of a Cholesterol Esterase Inhibitor from *Ganoderma lucidum*

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Bioassay-guided fractionation of a methanol extract of *Ganoderma lucidum* gave a pure cholesterol esterase inhibitor. On the basis of spectroscopic analysis and comparison with data from the literature, the structure of this compound was identified as 5α,8α-epidioxyergosta-6,22-dien-3β-ol (compound I). This compound inhibited cholesterol esterase activity with an IC_{50} value of 42 μM. Lineweaver–Burk plot analysis revealed that compound I is a noncompetitive inhibitor. The findings of this study suggest that compound I may be the active principle of the hypocholesterolemic effect of *Ganoderma lucidum*.

Keywords: *Ganoderma lucidum*, ergosterol peroxide, cholesterol esterase inhibitor, hypocholesterolemic effect

Cholesterol absorption has a significant impact on plasma cholesterol levels [6]. However, the process of cholesterol absorption has yet to be completely defined at the molecular level. Dietary cholesterol consists of free and esterified cholesterol, the ratio of which depends on the source. The precise physiological function of cholesterol esterase (CEase; E.C. 3.1.1.13) in free cholesterol absorption has been controversial [5, 10]. However, the correlation between the absorption of dietary cholesteryl ester and CEase activity has been unequivocally established [1, 2]. Hydrolysis of cholesteryl ester to cholesterol by CEase in the intestinal lumen is an essential process in the absorption of dietary cholesteryl ester [9]. Indeed, findings from animal studies have shown that inhibitors of CEase efficiently limit the absorption of dietary cholesterol esters [13]. Direct evidence of the involvement of CEase comes from a report on the impaired absorption of cholesterol administered as cholesterol esters in CEase gene knockout mice [8]. CEase is also reported to be involved directly in the regulation of serum cholesterol level; the enzyme has a phospholipase A_2 activity in the hydrolysis of phosphatidyl choline to lysophosphatidyl choline, which is required for the formation of intestinal micelles that facilitate free cholesterol delivery [11, 18]. Thus, inhibitors of CEase may limit the bioavailability of dietary cholesterol derived from cholesterol esters, suggesting a worthwhile strategy for the treatment of hypercholesterolemia and atherosclerosis.

In the course of screening for cholesterol esterase inhibitors from natural sources, an active compound was isolated from the fungus *Ganoderma lucidum* (*G. lucidum*). This mushroom has been used in East Asia for the treatment of various diseases and has recently attracted much attention on account of its biological activities [3, 17]. The present paper describes the isolation and characterization of the CEase inhibitor from *G. lucidum.*

Dried fruiting bodies of *G. lucidum* from the Yangpyung area were purchased from a local Oriental medicine market in Seoul, Korea. Ground fruiting bodies were extracted with methanol at room temperature by maceration. The resultant extract showed potent cholesterol esterase inhibitory activity.

The enzyme assay was performed as previously described [7]. The convenient substrate *p*-nitrophenylbutyrate was used for the assay of enzyme activity. Assays were performed in sodium phosphate buffer (0.1 M, pH 7.0) containing 6 mM taurocholate and 10 mM *p*-nitrophenylbutyrate in the presence or absence of samples. Reactions were initiated by the addition of enzyme [porcine pancreatic cholesterol esterase (Sigma) 160 mU] and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of ethanol. Using a spectrophotometer, the amount of liberated *p*-nitrophenol was determined from the absorbance at 405 nm. Three sets of data were collected for each sample concentration. A molar extinction coefficient of 16,300 M^{-1} cm^{-1} for *p*-nitrophenol was used to calculate the amount of product formed. Percentage inhibition was calculated by the formula

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(A−B)/A×100, where A is the amount of *p*-nitrophenol resulting from enzymatic hydrolysis in the absence of the inhibitor, and B is that in the presence of the inhibitor. The mode of enzyme inhibition was determined from the Lineweaver−Burk plot.

Bioactivity-guided fractionation of the methanol extract of *G. lucidum* (1.5 kg) led to the isolation of compound I. Combined extracts were concentrated *in vacuo* to yield a brown residue (23 g), which was partitioned between water and ethyl acetate. The ethyl acetate layer was dried over anhydrous $MgSO_4$, evaporated, and subjected to silica gel column chromatography. The column was eluted with a solvent mixture of petroleum ether−ethyl acetate (9:1−3:1, stepwise) to yield 12 fractions. The active fraction (F7, 410 mg) was concentrated and further purified by Sephadex LH-20 column chromatography using a CH_3OH−$CHCl_3$ (1:1) mixture. The active compound I crystallized from methanol as white needles. Its purity was monitored by thin layer chromatography (TLC). The TLC plate (Kieselegel 60 F_{254}; Merck) was developed using *n*-hexane−ethylacetate [1:1 (v/v)] as a solvent system. A single pink spot with an R_f value of 0.3 was detected on the plate after spraying with 15% phosphotungstic acid in ethanol solution (w/v) and heating at 100°C for 5 min. In the EI−MS spectrum, a molecular ion at *m/z* 428 was observed, whereas fragments at *m/z* 410 and 396 were due to the loss of H_2O and O_2 from the molecular ion, respectively.

The physical and spectroscopic data of the inhibitory compound I were as follows: mp 172−173°C, UV λ_{max} ($CHCl_3$): 237 nm. EI−MS *m/z* (%): 428 [M^+] (10), 410 [M^+-H_2O] (12), 396 (100), 376 (13), 363 (50), 337 (25), and 251 (30). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.82 (3H, s), 0.82 (3H, d, *J*=6.7 Hz), 0.83 (3H, d, *J*=6.6 Hz), 0.87 (3H, s), 0.89 (3H, d, *J*=6.5 Hz), 1.00 (3H, d, *J*=6.7 Hz), 3.97 (1H, m), 5.12 (1H, dd, *J*=8.9 Hz), 5.22 (1H, dd, *J*=8.9 Hz), 6.21 (1H, d, *J*=8.5 Hz), and 6.47 (1H, d, *J*=8.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δc 135.3 (C-22), 135.0 (C-6), 132.1 (C-23), 130.6 (C-7), 82.1 (C-5), 79.4 (C-8), 66.4 (C-3), 56.2 (C-17), 51.7 (C-14), 51.1 (C-9), 42.8 (C-24), 44.6 (C-13), 39.8 (C-20), 39.4 (C-12), 37.0 (C-4), 37.0 (C-10), 34.7 (C-1), 33.1 (C-25), 30.1 (C-2), 28.7 (C-16), 23.4 (C-15), 20.9 (C-21), 20.7 (C-11), 20.0 (C-27), 19.7 (C-26), 18.2 (C-19), 17.6 (C-28), and 12.9 (C-18). The ^{1}H NMR spectrum showed signals due to six methyl groups: four secondary methyls [δ 0.82 (3H, d, *J*=6.7 Hz), 0.83 (3H, d, *J*=6.6 Hz), 0.89 (3H, d, *J*=6.5 Hz), and 1.00 (3H, d, *J*=6.7 Hz)] and two signals from tertiary methyl groups [δ 0.82 (3H, s), and 0.87 (3H, s)]. The ^{13}C NMR spectrum with ^{1}H NMR spectral data showed the presence of two disubstituted olefins [δ 130.6, 132.1, 135.0, and 135.3]. Olefin protons [δ 6.47, and 6.21] with *cis*-coupling (*J*=8.5 Hz) and two oxygenated quaternary carbons of δ 79.4 and 82.1 suggested the presence of a peroxide structure. On the basis of the spectroscopic evidence obtained and comparison with data from the literature [4, 12, 14], the active constituent was identified as 5α,8α-epidioxyergosta-6,22-dien-3β-ol (Fig. 1).

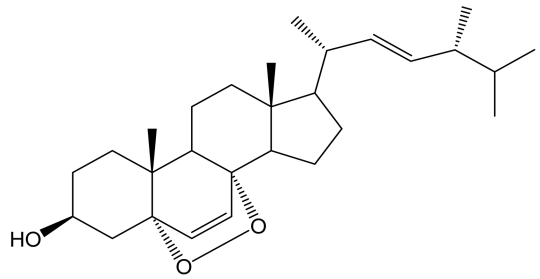

Fig. 1. Structure of the cholesterol esterase inhibitor from *G. lucidum*.

Compound I inhibited CEase in a concentration-dependent fashion with an IC_{50} of 42 μM (Fig. 2). When CEase was incubated with an amount of compound I capable of causing 90% inhibition of enzyme activity, followed by removal of the compound I on a PD 10 desalting column (Pharmacia, Piscataway, NJ, USA), nearly all of the enzyme activity was recovered. This result demonstrated that the inhibition of CEase by compound I was completely reversible. The inhibition mode of compound I was found to be noncompetitive by a Lineweaver−Burk double reciprocal plot of reaction velocity versus substrate concentration (Fig. 3), in which the K_m was the same at different inhibitor concentrations.

Previously, 5α,8α-epidioxyergosta-6,22-dien-3β-ol was isolated from various species of mushrooms, and its wide spectrum of biological effects, including antitumor, DNA topoisomerase I inhibitory, and immunosuppressive activities, has been demonstrated [15, 16]. However, the cholesterol

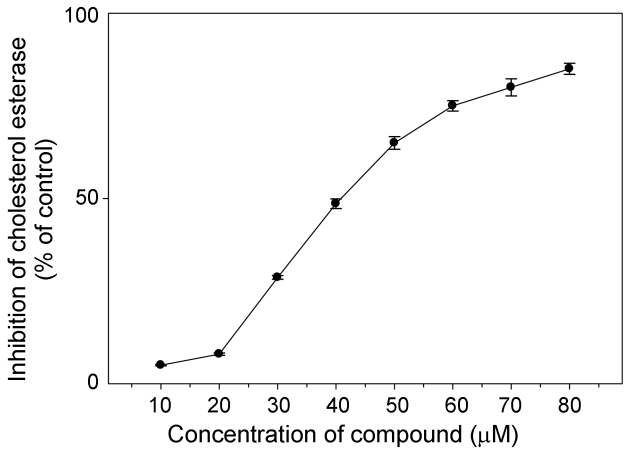

Fig. 2. Inhibitory effect of compound I on cholesterol esterase activity.
The reaction mixtures are described in the text. Inhibition of cholesterol esterase is expressed as a percent of enzyme control. Values are expressed as means±SD of triplicate reactions.

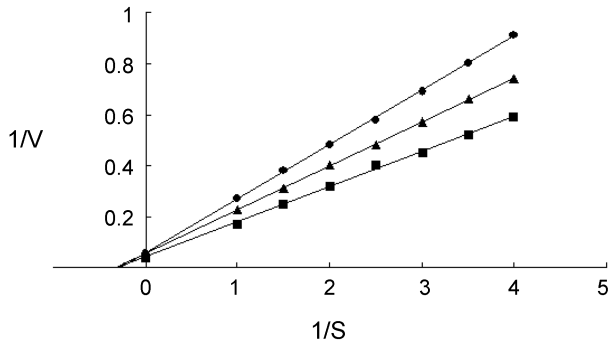

Fig. 3. Lineweaver–Burk plot analysis of the kinetics of cholesterol esterase inhibition by compound I.
The reaction mixture is described in the text. The concentration of compound I was 30 μM (–●–), 15 μM (–▲–), or 0 μM (–■–). Units are μmol/min for V and μM for S.

esterase inhibitory activity of this compound has not been previously reported. *G. lucidum* has received attention as a potential hypocholesterolemic agent [19]; however, the precise mechanism of this effect is as yet unknown. The results herein suggest that 5α,8α-epidioxyergosta-6,22-dien-3β-ol may be the active principle of the cholesterol-lowering effect of *G. lucidum.*

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