Effects of 32-Oxygenated Lanosterol Derivatives on 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity and Cholesterol Biosynthesis from 24,25-Dihydrolanosterol

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The effects of 32-oxygenated lanosterol derivatives on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and cholesterol biosynthesis from [24,25-^{3}H]24,25-dihydrolanosterol were studied. Among the derivatives, 3β-hydroxylanost-7-en-32-oic acid was the most active in depressing HMG-CoA reductase activity (IC_{50}: 0.7 μM) and cholesterol biosynthesis (IC_{50}: 0.4 μM) from 24,25-dihydrolanosterol.

Keywords 3-hydroxy-3-methylglutaryl coenzyme A reductase; cholesterol biosynthesis; lanosterol demethylation; 32-oxygenated lanosterol derivative; 24,25-dihydrolanosterol

Cholesterol is an important constituent of lipids and is biosynthesized from acetate *via* mevalonic acid, squalene, and lanosterol.[1] 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the formation of mevalonic acid is considered to be the rate-limiting enzyme in the sterol biosynthetic pathway. Many oxygenated sterols have been found to be potent inhibitors of sterol synthesis in animal cells in culture, and the main site of action appears to be at the level of HMG-CoA reductase.[2] However, mevalonic acid is a common precursor for all isoprenoids, and therefore blocking of mevalonic acid formation may induce undesired side effects other than inhibition of sterol synthesis. We have focused on lanosterol 14-demethylation and the subsequent steps leading to the formation of cholesterol.

We have reported the effects of lanosterol analogs,[3] cholesterol analogs,[4] oxygenated lanosterol derivatives,[5] and oxygenated cholesterol derivatives[6] on cholesterol biosynthesis from lanosterol or 24,25-dihydrolanosterol. From these studies, it was clarified that both the side chain and the skeleton structure of steroids are important for the inhibitory effects on cholesterol synthesis. Of the tested compounds, 7-oxolanost-8-en-3β-ol (7-oxo-DHL), 14α-methylcholest-7-ene-3β,15α-diol, and lanost-7-ene-3β,15α-diol have been shown to be potent inhibitors of cholesterol biosynthesis from lanosterol or 24,25-dihydrolanosterol in the hepatic subcellular fraction from rats, and the results suggested that the site of inhibition is lanosterol 14-demethylase (cytochrome $P\text{-}450_{14DM}$). 7-Oxo-DHL shows inhibitory activity on partially purified cytochrome $P\text{-}450_{14DM}$ from rat liver[7] and purified cytochrome $P\text{-}450_{14DM}$ from yeast.[8] Further, 7-oxo-DHL has been proved to exhibit hypolipidemic activity in rats.[9] In the hope of obtaining more potent derivatives of lanosterol, 32-oxygenated lanosterol derivatives (**1**—**5**, Fig. 1), including an antineoplastic sterol, 3β-hydroxylanost-7-en-32-oic acid,[10] were synthesized and their inhibitory activities on cholesterol biosynthesis from 24,25-dihydrolanosterol were determined. It was also considered of interest to determine in cell culture whether these steroids show an inhibitory effect on HMG-CoA reductase activity. In the present experiment, we defined the effect of lanosterol derivatives on HMG-CoA reductase activity using mouse L cells, and their inhibitory effects were compared with that of 25-hydroxycholesterol,[11] which is a well known HMG-CoA reductase inhibitor.

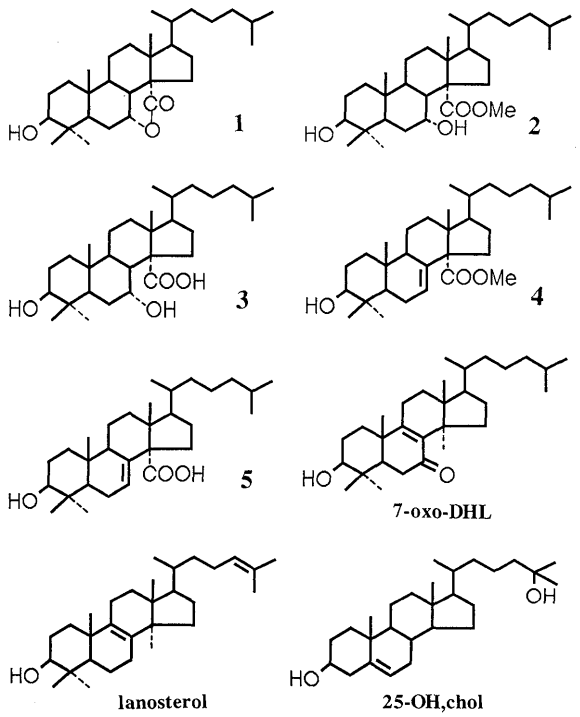

Fig. 1. Structures of Test Compounds

1: 3β-hydroxylanostano-32,7α-lactone (7,32-lactone), **2**: methyl 3β,7α-dihydroxylanostan-32-oate (7-OH, COOMe), **3**: 3β,7α-dihydroxylanostan-32-oic acid (7-OH, COOH), **4**: methyl 3β-hydroxylanost-7-en-32-oate (Δ^7-COOMe), **5**: 3β-Hydroxylanost-7-en-32-oic acid (Δ^7-COOH), 3β-hydroxylanost-8-en-7-one (7-oxo-DHL), 3β-hydroxylanosta-8,24-diene (lanosterol), 25-hydroxycholesterol (25-OH, chol).

Materials and Methods

Materials Lanosterol was obtained by column chromatography on 10% silver nitrate silica gel of commercial lanosterol (E. Merck, Darmstadt, Germany) which contained up to 35% (w/w) of 24,25-dihydrolanosterol. 7-Oxo-24,25-dihydrolanosterol was prepared as described previously.[12] Lanosterol derivatives were synthesized as described previously.[10] The purity of compounds used in this study was >98%. Dithiothreitol and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Boehringer Corporation (Mannheim, Germany). Thin-layer chromatography (TLC) was done using Merck precoated Kiesel gel plates (0.25 mm thick). [24,25-^{3}H]24,25-Dihydrolanosterol (39.59 MBq/mmol) was prepared by catalytic tritiation of lanosteryl acetate in the presence of 5% Pd–C at the Radiochemical Centre, Amersham, England, followed by alkaline hydrolysis as described previously.[5] DL-[3-^{14}C]HMG- CoA (1.92 GBq/mmol) and DL-[^{3}H]mevalonic acid (925 MBq/mmol) were obtained from New England Nuclear Corp (Wilmington, DE, USA). Brij 96 (detergent) was kindly supplied by Kao-Atras Co. (Japan). All other chemicals were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo). Fetal calf serum was purchased from GIBCO (U.S.A.). Dulbecco's modified Eagle minimum essential medium (DMEM) was obtained from Nissui Co. (Japan). Tissue culture dishes (60 × 15 mm) and flasks (75 cm^2, 250 ml) were obtained from Falcon Plastics (U.S.A.).

Incubation Procedure and Assays for Effects of Lanosterol Derivatives on Cholesterol Biosynthesis from 24,25-Dihydrolanosterol Hepatic subcellular 10000 × *g* supernatant (S-10) was prepared from Wistar male rats (125—150 g) as described by Bucher *et al.*[13] The incubation mixture consisted of S-10 fraction (4 ml, 22—23 mg protein/ml) and cofactors as described previously.[6] The control experiment was started by the addition of [24,25-^{3}H]24,25-dihydrolanosterol (2.21×10^5 dpm; 39.59 MBq/mmol, 18 μM) in phosphate buffer emulsion (0.1 ml) containing Tween 80 (3 mg). In the inhibition experiment, the substrate and lanosterol derivatives were added at the same time. Incubation was carried out at 37 °C for 3 h, and MeOH and KOH were added to final concentrations of 50% and 10%, respectively. The mixture was heated at 70 °C for 1 h, and sterols were extracted with CH_2Cl_2. The extracts were washed with water, dried over sodium sulfate and concentrated to a few milliliters (98% of added radioactivity was recovered). After addition of the carriers, 24,25-dihydrolanosterol (1 mg) and cholesterol (1 mg), to the solution, it was subjected to silica gel TLC. As standard samples, 24,25-dihydrolanosterol and cholesterol were also applied to both edges of the TLC plate to identify the zones of 4,4-dimethyl and 4,4-demethyl sterol fractions. After development with CH_2Cl_2 as the mobile phase, the radioactive 4,4-dimethyl sterol fraction and 4,4-demethyl sterol fraction were separated. Appropriate amounts of 24,25-dihydrolanosterol were added to the eluate of the 4,4-dimethyl sterol fraction and it was recrystallized several times to a constant specific activity. Appropriate amounts of cholesterol were added to the eluate of the 4,4-demethyl sterol fraction and it was recrystallized several times to a constant specific activity.

From the results, the yields of the recovered 24,25-dihydrolanosterol and 4,4-demethyl sterol (*i.e.* cholesterol) fractions were calculated. Results were expressed as the percentage inhibition as follows: percentage inhibition of cholesterol biosynthesis = [(percentage yield of cholesterol in control − percentage yield in run with test compound)/percentage yield in control] × 100.

Cell Culture Mouse L cells were grown as monolayers according to the method of Kaneko *et al.*[14] On day 0, 2.5×10^5 cells were seeded in 60 × 15 mm plastic dishes in DMEM containing 5% (v/v) fetal bovine serum. On day 3 or 4, logarithmically growing cells were treated with the test compound in 3 μl of EtOH for 3 or 24 h.

Assay of HMG-CoA Reductase Activity HMG-CoA reductase activity was assyed by the method of Kaneko *et al.*[14] Aliquots of each detergent-solubilized cell extract (40—50 μg of soluble protein) were incubated for 60 min in a final volume of 80 μl containing 0.1 M potassium phosphate buffer (pH 7.4), 2.5 mM NADPH, 4 mM dithiothreitol, 35 μM DL-[3-^{14}C]HMG-CoA (1.92 GBq/mmol). The reaction was terminated by adding 10 μl of 5 M HCl. After adding DL-[^{3}H]mevalonate as an internal standard the mixture was incubated at 37 °C for 30 min. The [^{14}C]mevalonolactone formed in each reaction was extracted with diethyl ether, isolated by TLC, and counted using an internal standard of [^{3}H]mavalonate to correct for incomplete recovery.

Results

Effects of Lanosterol Derivatives on Cholesterol Biosynthesis from 24,25-Dihydrolanosterol In the same way as described previously,[3-6] [24,25-^{3}H]24,25-dihydrolanosterol (18 μM) was incubated with the S-10 fraction of a rat liver homogenate in the presence of 32-oxygenated lanosterol derivatives (40 μM). The results are summarized in Table I. Among the tested compounds, Δ^7-COOH (**5**) was the most potent inhibitor (99% inhibition), and the inhibition was similar to that of 7-oxo-DHL (98% inhibition). However, Δ^7-COOMe (**4**) showed a moderate inhibitory effect, and 7,32-lactone (**1**) showed a low effect (27% inhibition). Although 7-hydroxy-COOH (**3**) also showed a high inhibitory effect (87% inhibition), 7-hydroxy-COOMe (**2**) had a low effect (32% inhibition). These results suggest that in the interaction of lanosterol derivatives with lanosterol 14-demethylase (cytochrome P-450$_{14DM}$), the carboxylic acid group makes a contribution. Further, Δ^7-COOH showed a higher inhibitory effect (99% inhibition) than 7-hydroxy-COOH (87% inhibition). This result suggests that the 7-double bond structure is more suitable than a saturated skeleton structure with 7-hydroxy group for producing the inhibitory activity. As Δ^7-COOH and 7-oxo-DHL showed the most potent inhibitory effect, these compounds were tested at lower concentrations (1, 5, 10 and 20 μM), and the results are shown in Fig. 2.

TABLE I. Cholesterol Biosynthesis during Incubation of the S-10 Fraction of Rat Liver Homogenate with [24,25-^{3}H]24,25-Dihydrolanosterol(DHL) in the Presence of the Oxygenated Sterols[a]

Compound (40 μM)	DHL (%)	Cholesterol (%)	Inhibition (%)
None	40.2	32.2	—
7,32-Lactone (**1**)	52.1	23.4	27
7-OH,COOMe (**2**)	55.0	21.8	32
7-OH,COOH (**3**)	83.5	4.2	87
Δ^7-COOMe (**4**)	67.5	16.3	49
Δ^7-COOH (**5**)	96.8	0.3	99
7-Oxo-DHL	95.8	0.6	98

a) [24,25-^{3}H]24,25-Dihydrolanosterol (2.21×10^5 dpm; 39.59 MBq/mmol, 18 μM) was incubated with rat liver S-10 fraction at 37 °C for 3 h. The incubation mixture contained, in a total volume of 5 ml, 4 ml of S-10 fraction and cofactors. Incubation was started by addition of the substrate and the test compound (40 μM) as an emulsion (0.1 ml) with Tween 80 (3 mg). Methods for analysis of incubation products and calculation of inhibition are described in Materials and Methods. Each value presents the mean of triplicate determination, and the S.E. of each value is less than 5% of the mean.

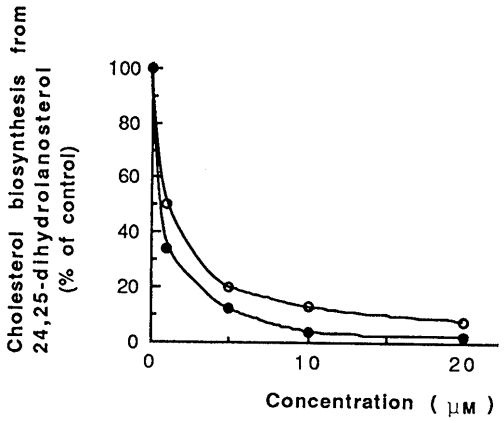

Fig. 2. Effects of 7-Oxo-DHL and Δ^7-COOH (**5**) on Cholesterol Formation from [24,25-^{3}H]24,25-Dihydrolanosterol

[24,25-^{3}H]24,25-Dihydrolanosterol was incubated with various concentrations of 7-oxo-DHL (○) and Δ^7-COOH (●). Data points represent the mean of triplicate determinations, and the S.E. of each point is less than 5% of the mean.

The concentrations required for 50% inhibition (IC_{50}) of these compounds were 0.4 and 1.0 μM for Δ^7-COOH and 7-oxo-DHL, respectively. Recently, Aoyama *et al.*[15] proposed a binding model of lanosterol and cytochrome P-450$_{14DM}$. From this model, it was thought that the COOH group on carbon 14 was suitable for interaction with the heme group of cytochrome P-450$_{14DM}$.

Effects of 32-Oxygenated Lanosterol Derivatives on HMG-CoA Reductase Activity in Mouse L Cells Since 32-oxygenated lanosterol derivatives (**1**—**5**) showed various rates of inhibition (27—99%) on cholesterol biosynthesis from 24,25-dihydrolanosterol, it was of interest to determine the effects of these compounds on HMG-CoA reductase activity. Lanosterol and 25-hydroxycholesterol were tested for comparison. These compounds inhibited HMG-CoA reductase activity upon treatment of mouse L cells for 3 h with various concentrations. Figure 3a shows the effects of **1**—**5**, lanosterol and 25-hydroxycholesterol. The inhibitory

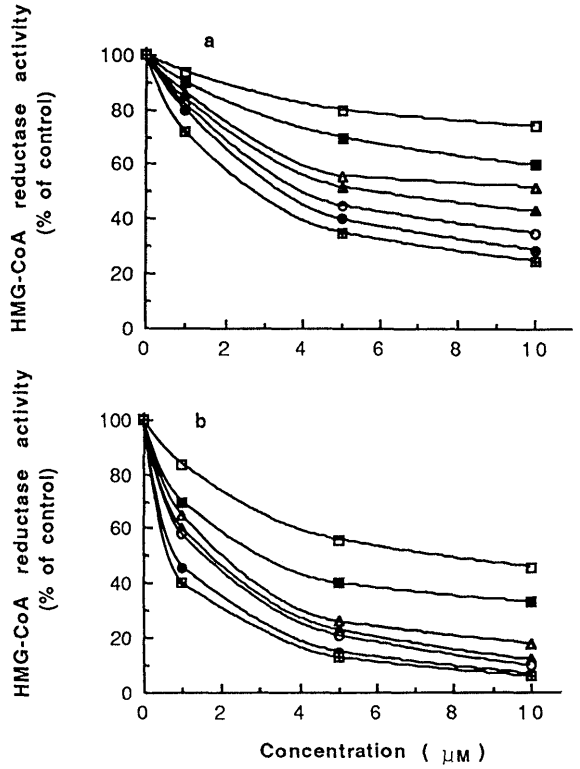

Fig. 3. Effects of Oxygenated Sterols on HMG-CoA Reductase Activity after Treatment for 3 h (a) or 24 h (b)

Mouse L cells were incubated with the oxygenated sterols; lactone (□), 7-OH, COOMe (■), lanosterol (△), Δ^7-COOMe (▲), 7-OH, COOH (○), Δ^7-COOH (●), and 25-hydroxycholesterol (⊞) at various concentrations. Data points represent the mean of triplicate determinations, and the S.E. of each point is less than 5% of the mean.

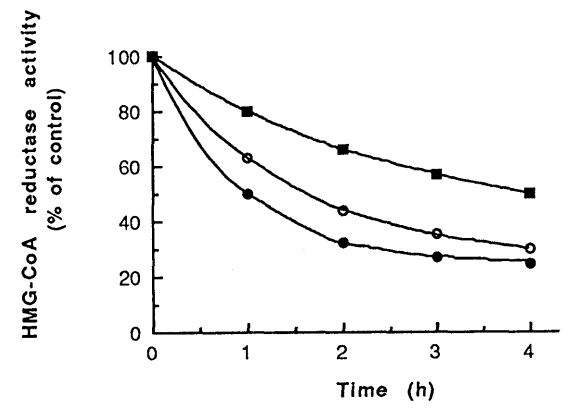

Fig. 4. Effects of Cycloheximide and Oxygenated Sterols on HMG-CoA Reductase Activity as a Function of Time

Mouse L cells were incubated with cycloheximide (■), lanosterol (○), and Δ^7-COOH (●) at 10 μM for various times. Data points represent the mean of triplicate determinations, and the S.E. of each point is less than 5% of the mean.

activities of these compounds were concentration-dependent.

When the cells were treated with these compounds for 24 h, the inhibition increased, as shown in Fig. 3b. The IC_{50} values after 24 h treatment are shown in Table II.

The kinetics of the decline of HMG-CoA reductase in the presence of potent inhibitors or cyloheximide are shown in Fig. 4. The half-life of the reductase in the presence of sterols (10 μM) was 60—100 min. This is considerably shorter than the value (240 min) in the presence of cycloheximide (10 μM). These results suggest that the sterols not only inhibit protein synthesis of the enzyme but also accelerate degradation of the enzyme protein or inactivate the enzyme by phosphorylation.

TABLE II. The Effects of Oxygenated Sterols on HMG-CoA Reductase Activity[a]

Sterol	IC_{50} (μM)[b]
7,32-Lactone (**1**)	7.4
7-OH,COOMe (**2**)	2.6
7-OH,COOH (**3**)	1.8
Δ^7-COOMe (**4**)	1.7
Δ^7-COOH (**5**)	0.7
25-OH,chol.	0.6
Lanosterol	2.0

a) Assays were carried out as described in Materials and Methods. *b*) Concentration required for 50% inhibition of mevalonic acid formation from HMG-CoA after 24 h treatment with each sterol.

Discussion

During cholesterol biosynthesis from 24,25-dihydrolanosterol in the presence of the lanosterol derivatives, the recovery yield of the substrate ([24,25-^{3}H]24,25-dihydrolanosterol) increased in parallel with the extent of inhibition. These results suggest that the lanosterol derivatives may inhibit the lanosterol 14-demethylase (cytochrome P-450_{14DM}), which is the enzyme acting at the first step of transformation of 24,25-dihydrolanosterol to cholesterol, as in the case[7,8] of 7-oxo-DHL. Recently we purified cytochrome P-450_{14DM} from pig liver microsomes,[16] and are planning the mechanistic study of the P-450_{14DM} with a potent inhibitor. The inhibitory effects of the lanosterol derivatives on HMG-CoA reductase activity after 3 or 24 h treatment were similar with those of these compounds on cholesterol biosynthesis from 24,25-dihydrolanosterol.

Recent investigations[17] have verified that the intermediates derived from lanosterol may also act as endogeneous regulators of reductase activity and sterol biosynthesis. Mayer *et al.*[18] reported that 3β-hydroxylanosta-8,15-diene-32-carboxylic acid inhibited HMG-CoA reductase activity and lanosterol 14-demethylase. Other sterols, *e.g.* 7α- and 7β-hydroxycholesterol, (22*R*)-hydroxydes-

The glass beads used in the present experiment showed cholest-7-ene-3β,15α-diol[21] have a similar effect. This work provides further evidence that certain oxygenated sterols inhibit cholesterol synthesis at two sites, one at the level of HMG-CoA reductase and the other at the level of the metabolism of lanosterol and 24,25-dihydrolanosterol. Chin *et al.*[22] reported that cholesterol appeared to enhance reductase degradation in cells by phosphorylation of reductase. This mechanism is also thought to explain the effects of these lanosterol derivatives on cholesterol biosynthesis. Gupta *et al.*[23] reported the effects of ketoconazole, a lanosterol 14-demethylase (cytochrome P-450_{14DM}) and another cytochrome P-450s inhibitor, on the regulation of HMG-CoA reductase activity in rat intestinal epithelial cell cultures. In that report, they proposed a schematic pathway showing the formation of endogenous oxysterols regulating HMG-CoA reductase. In our experiments also, endogeneous oxysterols may have been produced, suppressing HMG-CoA reductase activity.

It is important to continue the characterization of endogeneous oxysterols which would be formed in connection with the inhibition of lanosterol 14-demethylase, and to study further reductase degradation by lanosterol derivatives.

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