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Effects of Lanosterol Analogs on Cholesterol Biosynthesis from Lanosterol[1)]

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Cholesterol biosynthesis was examined in rat hepatic subcellular preparations (S-10) incubated with [24-^{3}H]-lanosterol in the presence of twelve synthetic lanosterol analogs with unnatural side chains (**1**—**12**), 20-iso-24-dihydrolanosterol, 25-hydroxy-24-dihydrolanosterol, 25-hydroxycholesterol and cyclolaudenol. Cholesterol biosynthesis from 18 μM [24-^{3}H]-lanosterol was inhibited by 40 μM lanosterol analogs. Among the analogs studied, 27-nordihydrolanosterol was most active in depressing cholesterol biosynthesis from lanosterol. The structure-activity relationship of lanosterol analogs and related compounds is discussed.

Keywords——cholesterol biosynthesis; [24-^{3}H]-lanosterol; inhibitor; lanosterol analogs; 20-iso-24-dihydrolanosterol; 25-hydroxy-24-dihydrolanosterol; rat hepatic subcellular preparations

The biosynthesis of cholesterol from lanosterol involves the removal of the three methyl groups,[2)] reduction of the Δ^{24}-double bond, and the migration of double bonds.[3,4)] However, very few studies[5)] have been carried out on the inhibition of cholesterol biosynthesis from lanosterol.

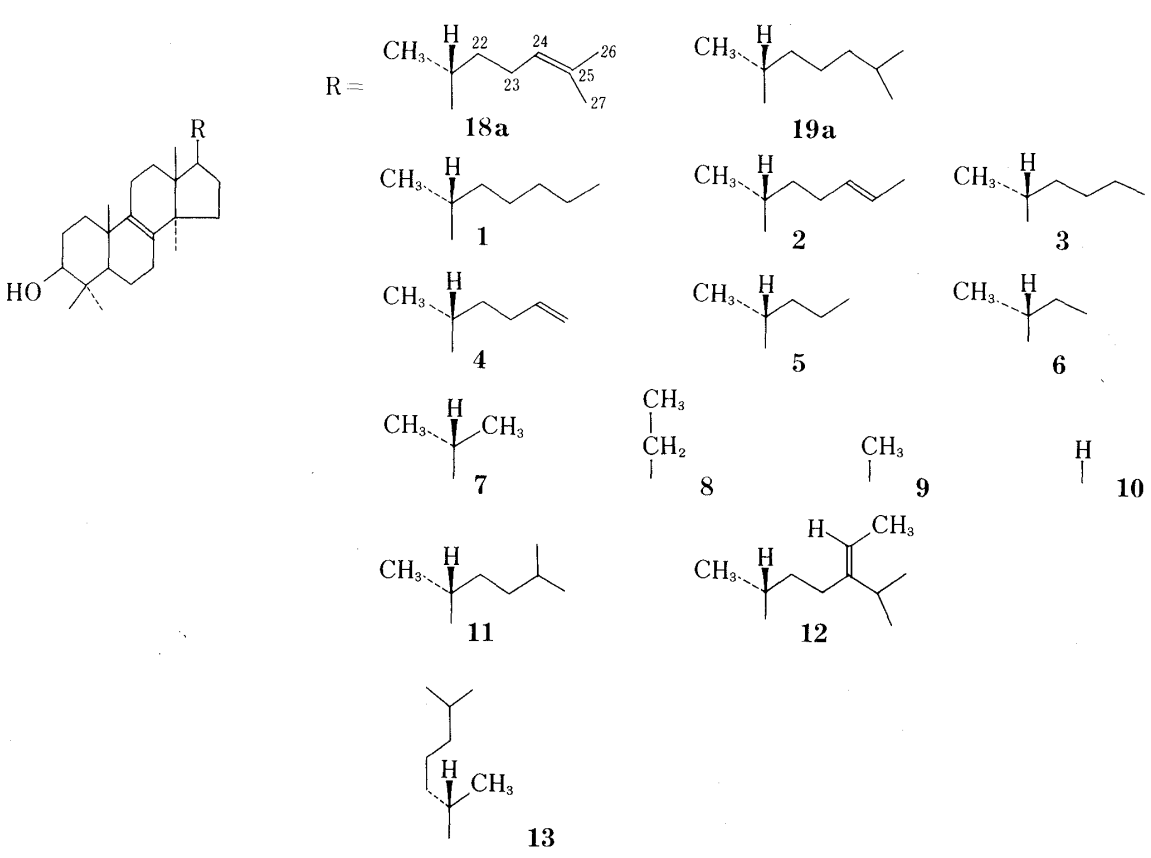

Fig. 1

Recently, we reported[1] the synthesis of various analogs of lanosterol (**1**—**13**) as shown in Fig. 1. This paper describes studies on the effects of these analogs on cholesterol biosynthesis from [24-^{3}H]-lanosterol in rat hepatic subcellular preparations (S-10) by the methods of Gibbons *et al.*[5,6] [24-^{3}H]-Lanosterol, the substrate of the biosynthetic experiments, was synthesized from 3β-acetoxylanost-8-en-24-one (**14**) as described in "Materials and Methods" (Chart 1).

Materials and Methods

Materials——Lanosterol and 24-dihydrolanosterol were obtained by column chromatography on 10% silver nitrate-impregnated silica gel of commercial lanosterol (E. Merck, Darmstadt, Germany) which contained up to 35% (w/w) of 24-dihydrolanosterol. Other lanosterol analogs were synthesized as described previously.[1] GSH, NADP$^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADH, NAD$^+$ and ATP were obtained from Boehringer Corporation (Mannheim, Germany). All other chemicals were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo). [^{3}H]-$NaBH_4$ (specific activity, 326 mCi/mmol) was purchased from New England Nuclear, Inc. (Boston). Silica gel for column chromatography was the product of Kanto Chemical Co., Inc. (Tokyo).

GLC and TLC——Gas liquid chromatography (GLC) was performed with a Shimadzu GC-6AF machine using a 1.5% OV-17 column. Thin-layer chromatography (TLC) was done on Merck precoated Kieselgel 60 plates (0.25 mm thick).

Synthesis of Unlabeled Lanosterol from 3β-Acetoxylanost-8-en-24-one (14) (Chart 1)——$NaBH_4$ (113.5 mg, 3 mmol) was added to a solution of **14** (484 mg, 1 mmol) in tetrahydrofuran (50 ml) and the mixture was stirred at room temperature for 4 days. Ice-water was added to this reaction mixture, and the whole was extracted with $CHCl_3$, washed with water, dried over sodium sulfate and evaporated to dryness. The residue was recrystallized from MeOH to give colorless needles of 3β-acetoxy-24ξ-hydroxylanost-8-ene (**15a**, 0.25 g), mp 149—151°C. *Anal.* Calcd for $C_{32}H_{54}O_3$: C, 78.96; H, 11.18. Found: C, 78.61; H, 11.08. MS *m/e*: 486 (M^+), 471, 453, 411 (M^+-75, base peak), 393. PMR δ (ppm): 0.69 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.02 (3H, s, 3β-$OCOCH_3$), 3.30 (1H, m, 24-H), 4.38—4.62 (1H, m, 3α-H).

$POCl_3$ (1 ml) was added to a solution of the 24-alcohol (**15a**, 0.25 g) in pyridine (5 ml), and the mixture was refluxed for 5 h. After cooling, the reaction mixture was poured slowly into ice-water and the mixture was extracted with $CHCl_3$, washed with 10% HCl and water, dried over sodium sulfate and evaporated to

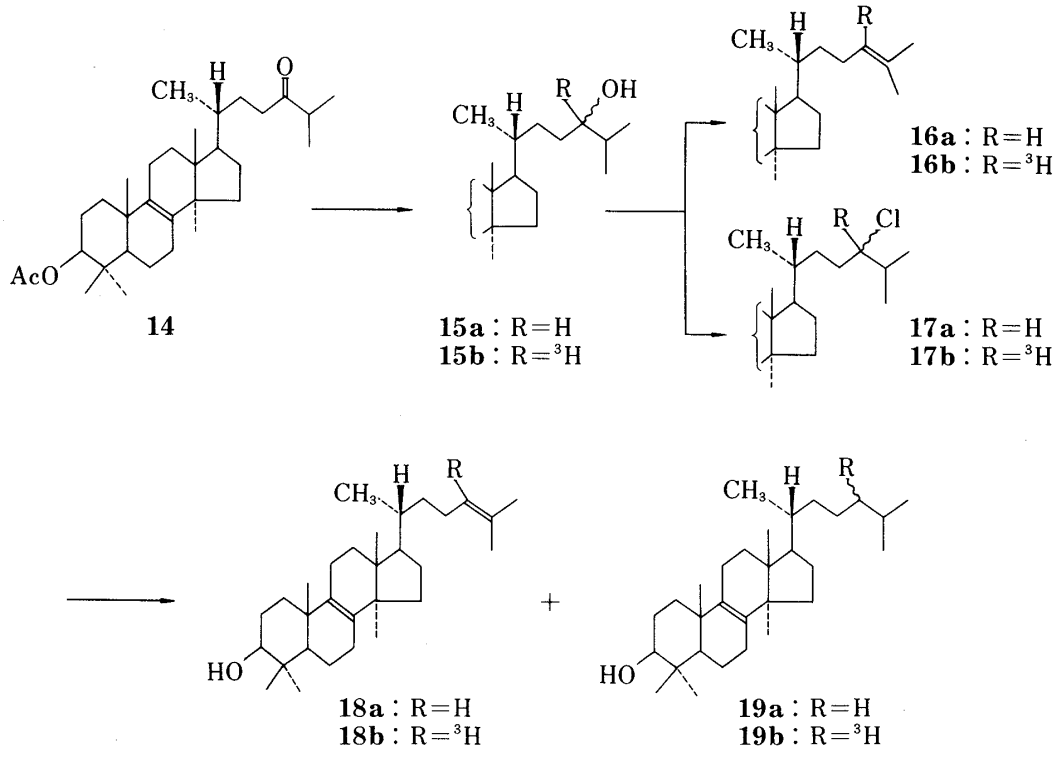

Chart 1

dryness. The residue was column chromatographed on silica gel (10 g). Elution with benzene gave a solid (0.16 g) which was a mixture of lanosteryl acetate (**16a**) (M^+, 468) and the 24-chloro compound (**17a**) (M^+, 504) in a ratio of 3: 1 as determined by GLC and PMR analyses.

An anhydrous dioxane (15 ml) solution of the mixture (**16a** and **17a**) was treated with $LiAlH_4$ (0.2 g), and the mixture was refluxed for 10 h. After cooling, the reaction mixture was poured slowly into ice-water, washed with water, dried over sodium sulfate and evaporated to dryness. The residue was column chromatographed on 10% silver nitrate-impregnated silica gel (40 g) and eluted with *n*-hexane–benzene (1: 1). The fractions were each analyzed by GLC with a 1.5% OV-17 column. Recrystallization of fractions 15—16 from MeOH gave colorless needles of 24-dihydrolanosterol (**19a**, 22 mg), mp 146—146.5°C, which was identical with an authentic sample. Recrystallization of fractions 19—20 from MeOH gave colorless needles of lanosterol (**18a**, 58 mg), mp 138—140°C, which was identical with an authentic sample.

Synthesis of [24-^{3}H]-Lanosterol (18b) (Chart 1)——**18b** was synthesized by the same method as described for the preparation of **18a**. [^{3}H]-$NaBH_4$ (1.5 mg, specific activity, 326 mCi/mmol) and $NaBH_4$ (112 mg) were added to a solution of the 24-ketone (**14**) (484 mg, 1 mmol) in tetrahydrofuran (50 ml) and the mixture was stirred at room temperature for 4 days. After work-up as described above, the crystalline 24ξ-hydroxy compound (**15b**) was treated with $POCl_3$ in pyridine to give a mixture of [24-^{3}H]-lanosteryl acetate (**16b**) and [24-^{3}H]-24ξ-chloro-24-dihydrolanosteryl acetate (**17b**). $LiAlH_4$ (0.2 g) was added to a dioxane solution (15 ml) of the mixture (**16b** and **17b**), and the whole was refluxed for 10 h. After work-up as described above, the residue was column chromatographed on 10% silver nitrate-impregnated silica gel (40 g). Elution with *n*-hexane–benzene (1: 1) gave a solid which was recrystallized from MeOH to give colorless needles of [24-^{3}H]-24-dihydrolanosterol (**19b**, 9 mg, 0.30 mCi/mmol). Further elution with benzene gave a solid which was recrystallized from MeOH to give colorless needles of [24-^{3}H]-lanosterol (**18b**, 24 mg, 0.43 mCi/mmol).

Synthesis of 25-Hydroxy-24-dihydrolanosterol——This material was synthesized by the method of Boar *et al.*[7] Recrystallization from MeOH gave colorless needles, mp 185—186°C (lit.[7] 184—186°C). PMR δ (ppm): 0.70 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.21 (6H, s, 26 and 27-CH_3), 3.25 (1H, m, 3α-H). MS *m/e*: 444 (M^+), 429, 426, 411 (M^+-33, base peak), 393.

Preparation of Rat Hepatic Subcellular Fraction and Incubation Procedure——Hepatic subcellular 10000×*g* supernatant fractions (S-10) were prepared from Wistar male rats weighing 125—150 g as described by Gibbons *et al.*[5,6] The incubation mixture consisted of S-10 fraction (4 ml, 17.5—17.8 mg protein/ml), 1 mM EDTA, 30 mM nicotinamide, 10 mM GSH, 2 mM $NADP^+$, 12 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 1.3 mM NADH, 0.8 mM NAD^+, 1.3 mM ATP, 4 mM $MgCl_2$ and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 5 ml. The control incubation was started by the addition of [24-^{3}H]-lanosterol in phosphate buffer emulsion (0.1 ml) containing Tween 80 (3 mg). In the inhibition experiments, [24-^{3}H]-lanosterol (18 μM) and lanosterol analogs or related compounds (40 μM each) were added at the same time in each experiment. In the preincubation experiments, 23,24,25,26,27-pentanordihydrolanosterol (**7**, 20 or 40 μM) was added to the incubation mixture and incubated at 37°C for 10 min. Then, a solution of [24-^{3}H]-lanosterol (18 μM) was added to allow its transformation to [^{3}H]-sterols. Incubations were 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 after which it was extracted with CH_2Cl_2 (20 ml × 2). The CH_2Cl_2 extracts containing tritium-labeled products were washed with water, dried over sodium sulfate, and concentrated to a few milliliters. After addition of carrier lanosterol (1.0 mg) to the solution, it was subjected to silica gel TLC with CH_2Cl_2 as the mobile phase. The 4,4-demethyl sterol fraction separated by silica gel TLC was isolated as the digitonin-precipitable sterols as described by Popják[8] and counted with a liquid scintillation spectrometer (Aloka LSC-502). The amount of cholesterol biosynthesis was determined from the radioactivity of the 4,4-demethyl sterol fraction (*i.e.*, cholesterol fraction).

Results and Discussion

When [24-^{3}H]-lanosterol (18 μM) was incubated with S-10 which had been preincubated with 23,24,25,26,27-pentanordihydrolanosterol (**7**) at a concentration of 20 μM, cholesterol biosynthesis was 82% inhibited. This pentanor compound completely halted cholesterol biosynthesis at a concentration of 40 μM (Table I).

Therefore, other lanosterol analogs and related compounds were tested at a concentration of 40 μM without preincubation. Among the tested compounds, 27-nordihydrolanosterol (**1**), which lacks the number 27 methyl group from the side chain of 24-dihydrolanosterol (**19a**), was the most potent inhibitor (81% inhibition) of cholesterol biosynthesis (Table II). On the other hand, 24-*trans*-27-norlanosterol (**2**), the 27-nor analog of lanosterol (**18a**), showed 78% inhibition. The 26,27-dinor analogs (**3** and **4**) and tri-, tetra- and pentanor analogs (**5**, **6** and **7**) of dihydrolanosterol also showed strongly inhibitory effects (47—67%). The hexa- and heptanor analogs (**8** and **9**) were considerably less inhibitory, and the octanor analog (**10**) showed no

Table I. Effect of Pentanordihydrolanosterol (7) on Cholesterol Biosynthesis from [24-^{3}H]-Lanosterol by the S-10 Fraction from Rat Liver (with Preincubation)

Concentration of the pentanor compound	Lanosterol Fr. (%)	Cholesterol Fr. (%)	Inhibition (%)
0 (control)	27.5	21.6	—
20 μM	81.3	3.8	82
40 μM	86.4	0	100

23,24,25,26,27-Pentanordihydrolanosterol (20 or 40 μM) in phosphate buffer emulsion (0.05 ml) containing Tween 80 (1.5 mg) was added to 4 ml of S-10 fraction with cofactors in a total volume of 5 ml and incubated at 37°C for 10 min. Then, [24-^{3}H]-lanosterol (90600 dpm; 0.43 μCi/μmol, 18 μM) emulsion (0.05 ml) containing Tween 80 (1.5 mg) was added and the mixture was incubated further at 37°C for 3 h. The control was started by the addition of [24-^{3}H]-lanosterol emulsion (0.05 ml) containing Tween 80 (1.5 mg) after preincubation for 10 min of the enzyme system with phosphate buffer (0.05 ml) containing Tween 80 (1.5 mg). The emulsion was prepared as follows: the substrate and the test compound were transferred to the flasks as acetone solutions and acetone containing Tween 80 was added. The solvent was evaporated off and the residue was suspended in 0.05 ml of phosphate buffer (pH 7.4). The incubation was terminated by the addition of potassium hydroxide–methanol solution. The radioactive 4,4-dimethyl sterol fraction and 4,4-demethyl sterol fraction were separated by silica gel TLC. Appropriate amounts of lanosterol were added to the eluate of the 4,4-dimethyl sterol fraction and it was recrystallized several times to constant specific activity. Results are expressed as the percentage inhibition as follows: Percent inhibition of cholesterol synthesis[9] = [(percent yield of cholesterol isolated by TLC in control − percent yield in run with test compound)/percent yield in control] × 10^2. Each incubation was carried out in duplicate and each value represents the average of the two experiments.

Table II. Cholesterol Biosynthesis during Incubation of S-10 Fraction of Rat Liver Homogenate with [24-^{3}H]-Lanosterol in the Presence of Lanosterol Analogs, Related Compounds, Cholesterol and 25-Hydroxycholesterol

Compound	Lanosterol Fr. (%)	Cholesterol Fr. (%)	Inhibition (%)
None (control)[a]	24.7	22.4	—
Lanosterol (L) (**18a**)	46.9	16.8	25
24-Dihydrolanosterol (DHL) (**19a**)	40.2	18.4	18
Cholesterol	47.6	17.4	22
27-Nor-DHL (**1**)	81.8	4.3	81
24-*trans*-27-Nor-L (**2**)	76.1	5.0	78
26,27-Dinor-DHL (**3**)	71.2	7.4	67
26,27-Dinor-L (**4**)	66.0	10.5	53
25,26,27-Trinor-DHL (**5**)	68.0	8.9	60
24,25,26,27-Tetranor-DHL (**6**)	65.3	11.0	51
23,24,25,26,27-Pentanor-DHL (**7**)	63.0	11.8	47
22,23,24,25,26,27-Hexanor-DHL (**8**)	37.4	19.9	11
21,22,23,24,25,26,27-Heptanor-DHL (**9**)	33.5	20.6	8
20,21,22,23,24,25,26,27-Octanor-DHL (**10**)	22.8	22.2	1
23-Nor-L (**11**)	60.4	10.8	52
[24(28)Z]-24-Ethylidene-DHL (**12**)	58.9	8.8	61
20-Iso-DHL (**13**)	33.5	19.5	13
25-Hydroxycholesterol	52.1	12.2	46
25-Hydroxy-DHL	71.8	7.6	66
Cyclolaudenol (**20**)	24.5	22.6	0

[24-^{3}H]-Lanosterol (90600 dpm; 0.43 μCi/μmol) 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 the addition of the substrate and test compounds as an emulsion (0.1 ml) with Tween 80 (3 mg). The concentration of [24-^{3}H]-lanosterol was 18 μM and that of the test compounds was 40 μM. Analytic methods for incubation products and the calculation of the percentage inhibition are described in Table I. DHL and L denote 24-dihydrolanosterol and lanosterol respectively.

a) [24-^{3}H]-Lanosterol wascon verted to 4α-methyl sterol (13.5%) and sterones (10.8%) other than cholesterol. The metabolism of [24-^{3}H]-dihydrolanosterol was similar to that of [24-^{3}H]-lanosterol.

activity. Therefore, the inhibitory activities of the analogs **1** to **10** increased with increase in the length of the side chain. In addition, 23-norlanosterol (**11**) and the [24(28)Z]-24-ethylidene compound (**12**) showed strongly inhibitory effects. However, cyclolaudenol[10] (24β-methyl-9,19-cyclolanost-25-en-3β-ol, **20**) (Fig. 2), whose ring imposes conformational changes as compared with lanosterol, showed no activity. These results indicated that the action of the analogs appears to be related to the conformation of the side chain and the skeleton. The addition of lanosterol (**18a**), which is the substrate itself, decreased the cholesterol formation to 16.8%. This can be explained as being due to the dilution of [24-^{3}H]-lanosterol (18 μM) by unlabeled lanosterol (40 μM). A similar effect occurs in the case of dihydrolanosterol (**19a**). However, the addition of cholesterol showed an inexplicable 22% inhibition in this experiment. Further, the 20-iso-24-dihydrolanosterol (**13**) showed 13% inhibition. This result indicates that the 20-iso compound (**13**), which has a different orientation in the side chain as compared with the 20-normal compound (**19a**), exhibited no apparent inhibitory activity. The addition of 25-hydroxycholesterol,[11,12] which is known to be a potent inhibitor of hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity in L cell culture,[13] caused 46% inhibition in this experiment. 25-Hydroxy-24-dihydrolanosterol showed a higher inhibitory effect than 25-hydroxycholesterol. In this connection, it is interesting that Kandutsch and his co-workers[14] found that two oxygenated lanosterol analogs, lanost-8-ene-3β,32-diol and 3β-hydroxylanost-8-en-32-al, inhibited the incorporation of acetate into sterols in cultures of Chinese hamster lung cells, mouse L-cells, and fetal mouse hepatic cells. Table II shows that, on the other hand, recovery yields of the starting material increase in parallel to the rates of inhibition. The above results show that lanosterol analogs, with both a side chain longer than that of the hexanor analog (**8**) and the C-20 normal configuration, were potent inhibitors of cholesterol biosynthesis, and suggest that these compounds may inhibit 14α-demethylation of lanosterol (which is a first step of transformation of lanosterol to cholesterol), though the S-10 fraction contains many enzymes. Recent work[15] has shown that 7α- and 7β-hydroxycholesterol and 22(*R*)-hydroxydesmosterol are inhibitors of lanosterol demethylation and HMG–CoA reductase activity in HTC cells.

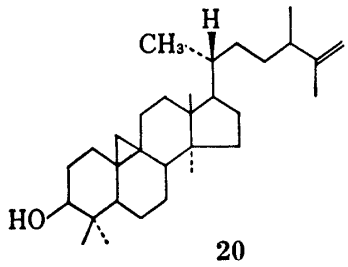

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Fig. 2

Since 27-nor-24-dihydrolanosterol inhibited the formation of [^{3}H]-cholesterol from [^{3}H]-lanosterol, it would be of interest to determine whether it is also a substrate or not. Studies on the metabolism of the 27-nor compound (**1**) will be reported in detail elsewhere.

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