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Metabolism of 32-Hydroxylated 24,25-Dihydrolanosterols by Partially Purified Cytochrome P-450$_{14DM}$ from Rat Liver Microsomes

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Metabolism of 32-hydroxylated 24,25-dihydrolanosterols (**1**—**3**), including the intermediate of lanosterol and 24,25-dihydrolanosterol (**4**, DHL) demethylation, were studied in a reconstituted system consisting of rat liver partially purified cytochrome P-450, which catalyzes lanosterol 14-demethylation (cytochrome P-450$_{14DM}$), and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase. The reconstituted system converted lanost-8-ene-3β,32-diol (**1**) to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**5**), the 14-dehydroxymethylated product, in the same way as **4**. Lanost-7-ene-3β,32-diol (**2**) and lanost-6-ene-3β,32-diol (**3**), the isomers of **1**, were not converted to the corresponding 14-dehydroxymethylated products. The apparent K_m value of cytochrome P-450$_{14DM}$ for **1** was about 1/6 of that for **4**. The metabolism of **4** was inhibited by 7-oxo-24,25-dihydrolanosterol (**6**, 7-oxo-DHL), which is a potent inhibitor of cholesterol biosynthesis from lanosterol or **4**. However, the metabolism of **1** was less inhibited by **6** than that of **4**.

Keywords——lanosterol 14-demethylation; cytochrome P-450; 32-hydroxylated 24,25-dihydrolanosterol; 7-oxo-24,25-dihydrolanosterol

Introduction

We have previously studied[1] the effects of many oxygenated 24,25-dihydrolanosterols on cholesterol biosynthesis from 24,25-dihydrolanosterol in rat liver S-10 fraction. Among these compounds, 7-oxolanostene derivatives showed potent inhibitory effects, and it was suggested that they would interact with cytochrome P-450, which catalyzes lanosterol 14-demethylation (cytochrome P-450$_{14DM}$). Compounds **1**—**3** showed moderate inhibitory effects. This finding prompted us to determine whether **1**—**3** can be metabolized as substrates and whether an effective inhibitor (7-oxo-24,25-dihydrolanosterol(7-oxo-DHL))[2] can show an inhibitory effect on the demethylation system reconstituted with cytochrome P-450$_{14DM}$ and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-P-450-reductase. For this purpose, purification of cytochrome of P-450$_{14DM}$ of rat liver microsomes was essential. Cytochrome P-450$_{14DM}$ in mammalian microsomes has been purified by Iwasaki *et al.*[3] and Trzaskos *et al.*,[4] but, its substrate specificity has not been studied. On the other hand, cytochrome P-450$_{14DM}$ of yeast has been purified by Yoshida and co-workers[5,6] from *Saccharomyces cerevisiae* microsomes and characterized in detail.

The initial step of sterol biosynthesis from lanosterol (lanosta-8,24-dien-3β-ol) in yeast and mammals is oxidative removal of the 14-methyl group (C-32) of lanosterol catalyzed by a cytochrome P-450-containing enzyme system. The 14-methyl group is removed as formic acid and lanosterol is converted to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. This compound is further converted to cholesterol *via* several steps (Fig. 1). Compound 4 is also demethylated like lanosterol, and converted to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**5**).

It seemed of interest to test **1**—**3** as substrates of cytochrome P-450$_{14DM}$ of rat liver

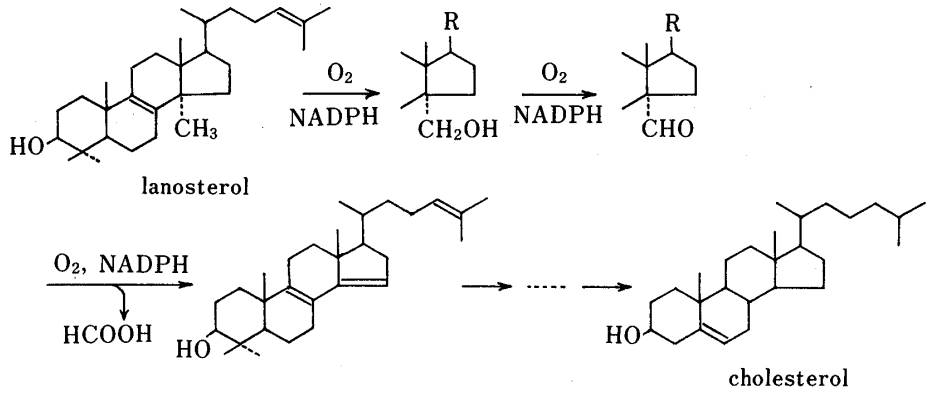

Fig. 1. Hypothetical Pathway for 14α-Demethylation of Lanosterol or DHL to Cholesterol

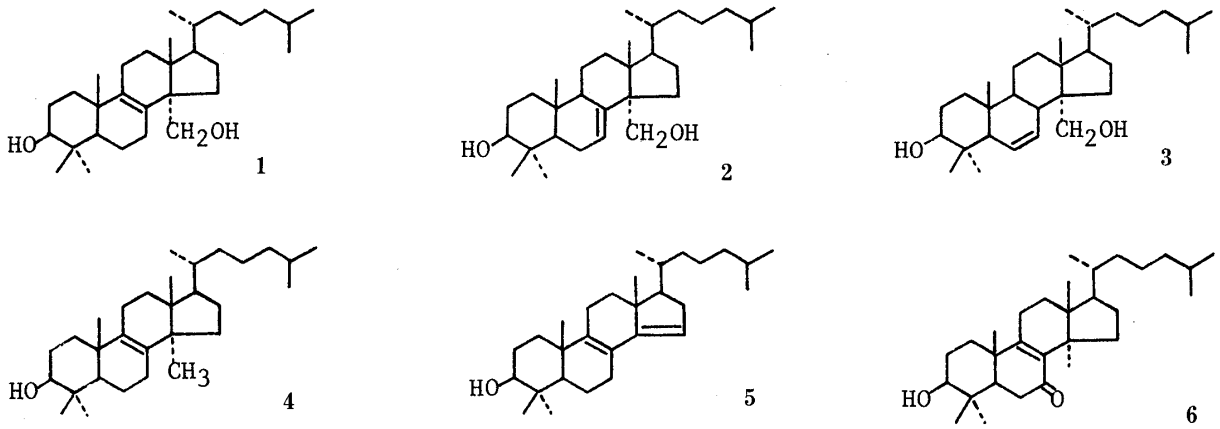

Fig. 2. The Structures of Compounds **1**—**6**

microsomes, since studies on double bond isomers will give information on the mode of interaction between cytochrome P-450_{14DM} and its substrates. In this experiment, **1**, **2**, and **3** were used as substrates. Only the Δ^8 derivative was converted to the 14-dehydroxymethylated product (**5**). Further, the effect of 7-oxo-DHL on the metabolism of **4** and **1** was studied.

Experimental Procedures

Materials——Lanost-8-ene-3β,32-diol (**1**), lanost-7-ene-3β,32-diol (**2**) and lanost-6-ene-3β,32-diol (**3**) were synthesized as described previously.[7] 4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol (**5**) was synthesized by the method of Paik *et al.*[8] DHL (**4**) was prepared by hydrogenation of a commercial mixture of lanosterol and 24,25-dihydrolanosterol. 7-Oxo-DHL (**6**) was prepared by the method of Pinky *et al.*[9] Dilauroyl phosphatidyl choline (DLPC) was obtained from Sigma Chemical Co. Other chemicals and biochemicals used were of the highest quality available commercially.

Enzyme Preparation——Partially Purified Cytochrome P-450_{14DM}: The procedure followed the method of Iwasaki *et al.*[3] Microsomes were prepared from livers of male Wistar rats (220—230 g). Microsomes (specific content of cytochrome P-450: 0.76 nmol/mg protein) were solubilized with sodium cholate and the solubilized supernatant fraction was chromatographed on an aminooctyl-Sepharose column. The cytochrome P-450 fraction eluted with 10 mM potassium phosphate buffer (KPB) was subjected to DE-52 column chromatography at room temperature with a linear gradient of NaCl (0—180 mM) and the initially eluted cytochrome P-450 fraction was subjected to hydroxyapatite column chromatography. The cytochrome P-450 fraction eluted with 200 mM KPB was collected and the Emulgen was removed by stirring with Biobeads. The specific content of the final enzyme was 4.12 nmol/mg protein. Protein was determined by the method of Lowry *et al.*[10] using bovine serum albumin as a standard.

NADPH-Cytochrome P-450 Reductase: This enzyme was purified from rat liver microsomes according to the method of Yasukochi and Masters.[11]

Assay for 14-Demethylation by the Reconstituted System——The substrate was dispersed with DLPC as described by Aoyama *et al.*[12] Then 30 μl of cytochrome P-450 (0.18 nmol) in 0.1 M KPB (pH 7.4) containing 20% glycerol, 5 μl of NADPH-cytochrome P-450 reductase (1 unit) in 10 mM KPB (pH 7.7), and 5 μl of the substrate solution dispersed with DLPC were mixed and sonicated, and 0.36 ml of 0.1 M KPB (pH 7.4) containing glucose-6-phosphate (final 40 mM), $MgCl_2$ (final 0.4 mM) and glucose-6-phosphate dehydrogenase (0.2 unit) was added. The reaction was started by adding 0.1 ml of NADPH solution (final 2 mM) and incubation was carried out at 37 °C in air.

Analytical Methods——The reaction was stopped by adding 2 ml of 20% (w/v) KOH and 1.5 ml of MeOH. The reaction mixture was saponified at 80 °C for 1 h. Sterols were extracted with CH_2Cl_2 and the organic layer was dried over sodium sulfate. After evaporation of the solvent, a portion of the product was trimethylsilylated. The trimethylsilylated sterols were analyzed with a JEOL gas chromatograph-mass spectrometer equipped with a 10 m × 0.2 mm SP-2250 fused silica capillary column (Supelco Inc.), with helium as a carrier gas. Samples were injected at an initial column temperature of 50 °C. After 4 min, the temperature was raised to 255 °C at a rate of 32 °C/min. The injector, separator, and inlet temperatures were 270, 255, and 260 °C, respectively. On the other hand, sterols extracted from the reaction mixture were analyzed by high-performance liquid chromatography (HPLC). HPLC was performed on a μBondapak C_{18} reverse-phase column (3.9 mm × 30 cm), using a Waters pump (model 510) and a Waters detector (model 480 spectrometer, set at 214 or 248 nm). Acetonitrile–methanol–water (45 : 45 : 10, v/v/v) was used as an eluent (flow rate 1.0 ml/min). For the calculation of the activity (nmol of product formed/min), ergosterol (5 μg) was added as an internal standard before extraction of the incubation mixture and HPLC analysis (248 nm). The activity was calculated from the areas of the two peaks (retention times: ergosterol, 16.8 min; product, 24.6 min).

Results and Discussion

Metabolism of 24,25-Dihydrolanosterol and 32-Hydroxylated 24,25-Dihydrolanosterols

The reconstituted system consisting of partially purified cytochrome $P\text{-}450_{14DM}$ and NADPH-cytochrome P-450 reductase catalyzed the 14-demethylation of **4**. Figure 3B represents the gas-chromatographic (GC) detection of **4** and its metabolite. The metabolite, which had a relative retention time with respect to **4** of 1.04, was identified as 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**5**) by comparison with an authentic sample (Table I) and this compound was the sole metabolite formed from the substrate by the reconstituted system under these conditions.

Next, the metabolism of 32-hydroxylated 24,25-dihydrolanosterols (**1**—**3**) was studied. Figure 3C shows a gas chromatogram of **1** and its metabolite. It is clear from the

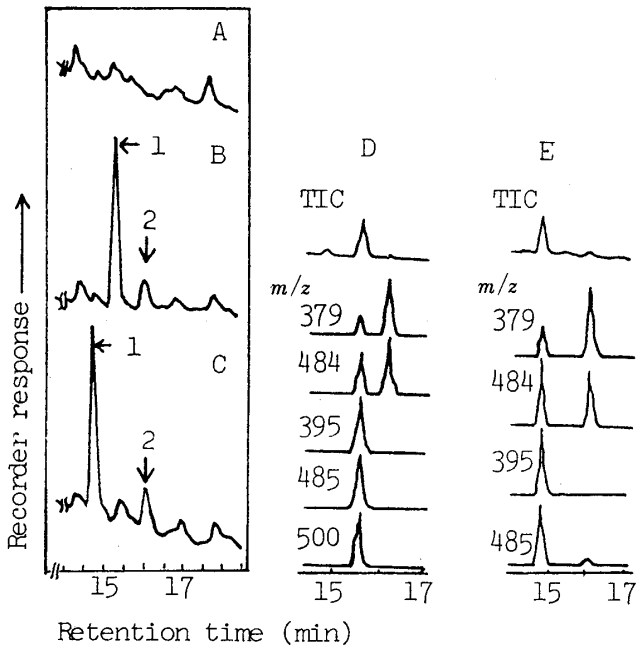

Fig. 3. GC Detection of the Metabolite of **4** and **1**

Panel A: Incubation was carried out without substrate as described for panel B. Extract in the reaction mixture was analyzed as described bellow. Panel B: **4** (23 nmol) dispersed with DLPC (50 μg) was incubated with the reconstituted system consisting of 0.18 nmol of partially purified $P\text{-}450_{14DM}$ and 1.0 unit of NADPH-cytochrome P-450 reductase in a reaction mixture (0.5 ml) consisting of 2 mM NADPH, 40 mM glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase and 0.1 M potassium phosphate buffer (pH 7.5). The reaction mixture was incubated at 37 °C for 30 min in air. Sterols extracted from the reaction mixture were trimethylsilylated and analyzed by gas chromatography-mass spectrometry with an SP-2250 capillary column. Peaks 1 and 2 represent **4** and **5**. Panel C: **1** (23 nmol) dispersed with DLPC (50 μg) was incubated as above. Sterols in the reaction mixture were analyzed as above. Peaks 1 and 2 represent **1** and **5**, respectively. Panel D: Mass chromatogram of the product in panel B. The column effluent was monitored by mass chromatography at *m/z* 379, 484, 395, 485, and 500 as well as measuring the total ion current (TIC). Panel E: Mass chromatogram of the product in panel C. The column effluent was monitored by mass chromatography at *m/z* 379, 484, 395, and 485 as well as by measuring the total ion current.

Table I. Mass Spectra of Trimethylsilyl Derivatives of DHL, Δ^8-32-Hydroxylated Compound and Their Metabolites

Ion species	Trimethylsilylated DHL		Ditrimethylsilylated Δ^8-CH_2OH[a]		Trimethylsilylated metabolite from DHL		Trimethylsilylated metabolite from Δ^8-CH_2OH[a]	
	m/z	Intensity	*m/z*	Intensity	*m/z*	Intensity	*m/z*	Intensity
M^+	500	29.0	—	—	484	56.1	484	42.0
M^+-CH_3	485	46.0	573	4.2	469	3.5	469	5.4
$M^+-TMSOH$	—	—	498	4.0	394	8.8	394	17.2
M^+-CH_2OTMS	—	—	485	85.0	—	—	—	—
$M^+-CH_3-TMSOH$	395	100.0	—	—	379	100.0	379	100.0
$M^+-CH_2OTMS-TMSOH$	—	—	395	100.0	—	—	—	—

a) Δ^8-CH_2OH represents lanost-8-ene-3β,32-diol (**1**).

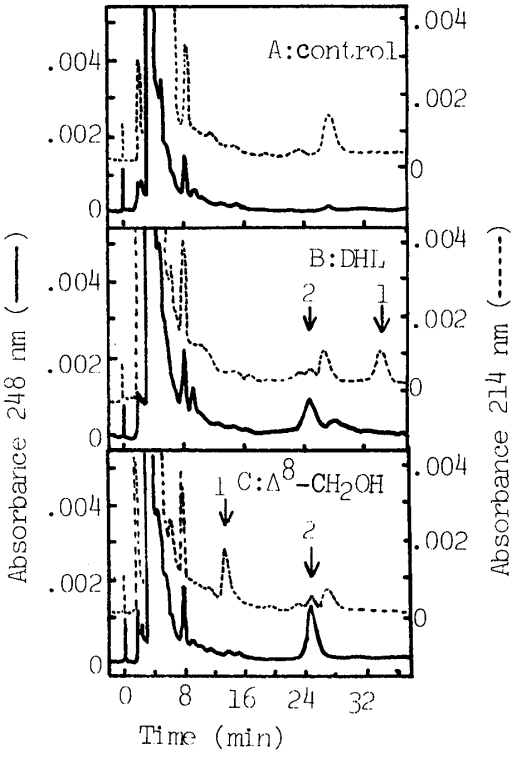

Fig. 4. Reverse-Phase HPLC with Detection at 214 and 248 nm of the Metabolite from **4** and **1** Formed by the Reconstituted System

Panel A: The reaction extract without substrate. Panel B: Sterols in the reaction extract of **4**. Panel C: Sterols in the reaction extract of **1**.

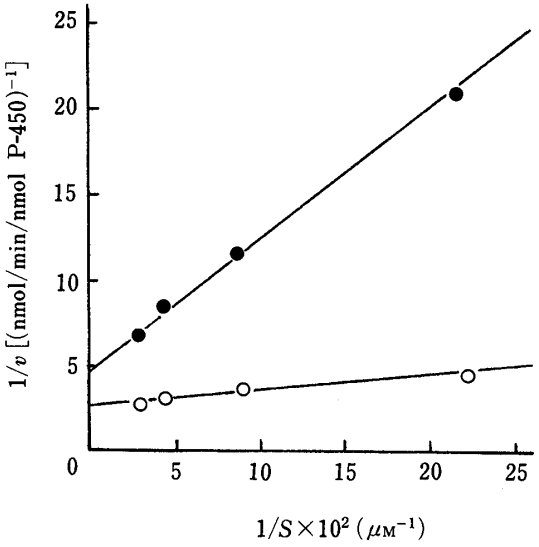

Fig. 5. Double-Reciprocal Plots of **4** and **1** Metabolism by the Reconstituted System

Metabolism of **4** and **1** was assayed as described in the legend to Fig. 3 except that the concentrations of the substrates were varied as indicated. (●), DHL; (○), Δ^8-32-hydroxylated compound.

chromatograms that **1** was converted to a metabolite which showed the same GC behavior as that of **4**, and its mass spectrum coincided with that of an authentic sample as shown in Table I.

Figure 3D shows a mass chromatogram of **4** and the metabolite. The ions at *m/z* 484 and 379 can be identified as the molecular ion (M^+) and $M^+-CH_3-TMSOH$ of the 14-demethylated product (**5**). The ions at *m/z* 500, 485, and 395 can be identified as M^+,

M^+-CH_3, and $M^+-CH_3-TMSOH$ of **4**. Figure 3E shows a mass chromatogram of an extract from **1** and the metabolite. The ions at *m/z* 484 and 379 were identified in the same way as described in the case of **4** and its metabolite. The ions at *m/z* 485 and 395 can be identified as M^+-CH_2OTMS and $M^+-CH_2OTMS-TMSOH$ of **1**. However, **2** and **3** were not converted to the corresponding 14-dehydroxymethylated products and no other metabolite was detected on GC. The position of the double bond thus appears to have a strong influence on the removal of the 14-hydroxymethyl group of 32-hydroxylated 24,25-dihydrolanosterols. Further, the metabolite generated by the reconstituted system can be detected by HPLC assay (Fig. 4). Figure 4B and 4C show the sterols in the reaction extract of **4** and **1**. Figure 4A shows the reaction extract without substrate. Detection at 248 nm presents unambiguous evidence for the presence of an 8,14-diene system. It can be concluded that the reconstituted system catalyzes the removal of the 14-hydroxymethyl group of **1** as well as the 14-methyl group of **4**, and both of these sterols are converted to the same metabolite (**5**). Aoyama *et al.*[12] recently proved that **1** is converted to the 14-demethylated product by cytochrome $P\text{-}450_{14DM}$ from *Saccharomyces cerevisiae*. Further, they demonstrated that cytochrome $P\text{-}450_{14DM}$ catalyzes all the processes of the demethylation, consisting of three monooxygenations. The present results with mammalian cytochrome $P\text{-}450_{14DM}$ suggest that the processes of demethylation are the same as in the case of *S. cerevisiae* cytochrome $P\text{-}450_{14DM}$.

As shown in Fig. 5, the apparent K_m value of the reconstituted system for **1** was determined as 3.3 μM while that for 4 was 19.2 μM. These results indicated that cytochrome $P\text{-}450_{14DM}$ showed higher affinity for **1** than for **4**.

Effect of 7-Oxo-DHL on the Metabolism of the Δ^8-32-Hydroxylated Compound and DHL

Aoyama *et al.*[13] reported recently that 7-oxo-DHL (**6**) has two characteristics as a lanosterol 14-demethylase inhibitor: it is a typical competitive inhibitor, and it acts as an inhibitor of electron transfer to the oxyferro intermediate. In this experiment, **6** inhibited the removal of the 14-methyl group from DHL by the reconstituted system, as shown in Fig. 6. Compound **6** also inhibited the removal of the 14-hydroxymethyl group from **1**, but the inhibitory effect was weaker than that in the case of **4**. These results reflect a higher affinity of **1** for cytochrome $P\text{-}450_{14DM}$, and further suggest that **1** is held at the active site of the

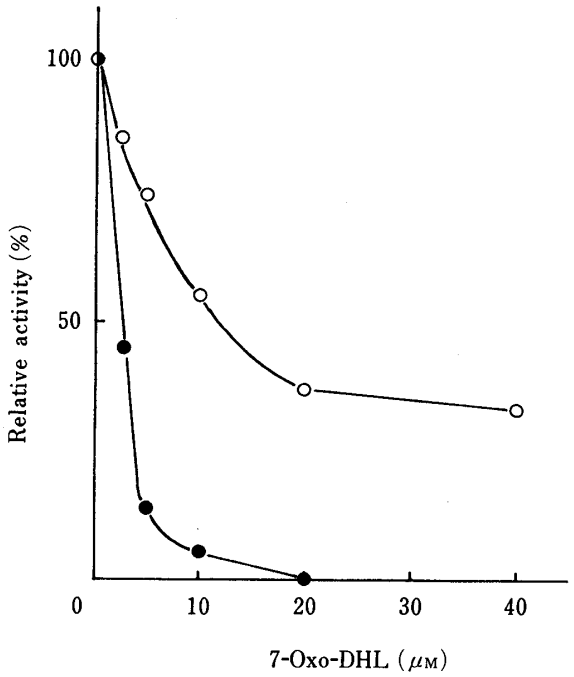

Fig. 6. Effect of **6** on the Metabolism of **4** and **1** by the Reconstituted System

Metabolism of **4** and **1** was assayed as described in the legend to Fig. 3 in the presence of the indicated concentrations of **6**. Compound **6** was added to the reaction mixture as a DLPC solution (5 μl). A corresponding volume of the solvent was added in the control experiment. (●), DHL; (○), the Δ^8-32-hydroxylated compound.

cytochrome and not released. The inhibitory effect of **6** on **1** would originate from its second inhibitory mode, inhibition of electron transfer to the oxyferro intermediate, as clearly demonstrated with yeast cytochrome P-450_{14DM}.[12]

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