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

Yoshiko SONODA, Yoshio SEKIGAWA, and Yoshihiro SATO*

Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105, Japan. *Received July 12, 1988*

27-Nor-24,25-dihydrolanosterol (27-nor-DHL), 26,27-dinor-24,25-dihydrolanosterol (26,27-dinor-DHL), and 25,26,27-trinor-24,25-dihydrolanosterol (25, 26,27-trinor-DHL), analogs of 24,25-dihydrolanosterol (DHL) which have no C-27 carbon, C-26,27 carbons and C-25,26,27 carbons, were converted to the corresponding 14-demethylated products using a reconstituted monooxygenase system from rat liver microsomes which contained cytochrome P-450$_{14DM}$ catalyzing lanosterol 14-demethylation and reduced nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase in the presence of NADPH and molecular oxygen. Each metabolite showed a relative retention time (Rt_R) of 0.72 with respect to each substrate in high-performance liquid chromatography (HPLC) on a reversed-phase column. Comparison of each gas chromatography-mass spectrum and Rt_R value with those of the metabolite of DHL, 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, indicated that the metabolites could be inferred to be 27-nor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, 26,27-dinor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, and 25,26,27-trinor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol. However, 24,25,26,27-tetranor- and 23,24,25,26,27-pentanor analogs of DHL and 20-iso-24,25-dihydrolanosterol were not metabolized by the reconstituted enzyme system.

Keywords lanosterol 14-demethylation; cytochrome P-450; 27-nor-24,25-dihydrolanosterol; 26,27-dinor-24,25-dihydrolanosterol; 25,26,27-trinor-24,25-dihydrolanosterol; 7-oxo-24,25-dihydrolanosterol

The initial step in sterol biosynthesis from lanosterol in yeast and mammals is the oxidative removal of the 14-methyl group (C-32) catalyzed by a cytochrome P-450 (cytochrome P-450$_{14DM}$).[1] The 14-methyl group is removed as formic acid *via* the Δ^8-32-hydroxy compound. 24,25-Dihydrolanosterol (**1a**, DHL) is also demethylated as well as lanosterol, and is converted to the 8,14-diene compound (**2a**).

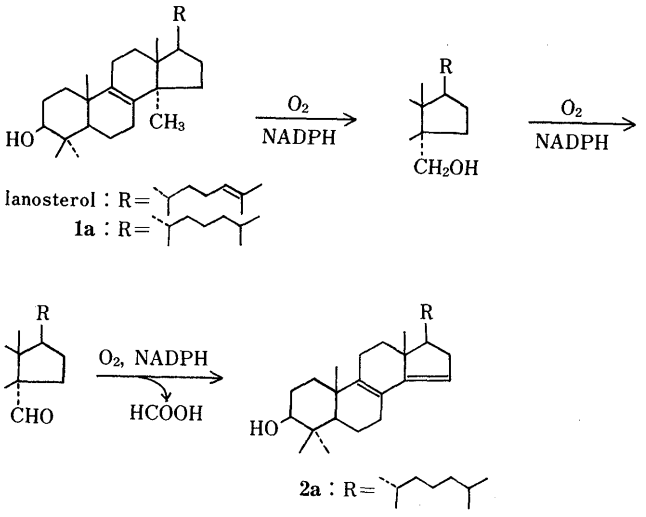

Chart 1

We demonstrated recently[2] that the substrate specificity of partially purified cytochrome P-450$_{14DM}$ from rat liver microsomes is high for the double bond position on the steroid nucleus (Chart 2). That is, the reconstituted system containing partially purified cytochrome P-450$_{14DM}$ converted the Δ^8-32-hydroxylated compound (**3**) to **2a**, but the isomers of **3**, Δ^6-32-hydroxylated and Δ^7-32-hydroxylated compounds, were not converted by the same reconstituted system to the corresponding dehydroxymethylated products. However, the substrate specificity of P-450$_{14DM}$ for the steroid side chain is not known. Studies on the substrate specificity of P-450$_{14DM}$ for the steroid side chain and nucleus may afford important information concerning the interaction between the substrate and P-450$_{14DM}$. In the present experiments, we used as substrates DHL analogs (**1b**—**1f**) and 20-iso-DHL (**4**), which have unnatural side chains as illustrated in Fig. 1. We demonstrated previously that the DHL analogs (**1b**—**f**) are potent inhibitors of cholesterol biosynthesis from lanosterol.[3] Further, it was found that 27-nor-DHL (**1b**) was transformed to the cholesterol analog, 27-norcholesterol, but 23,24,25,26,27-pentanor-DHL (**1f**) was not transformed to the corresponding cholesterol analog by rat liver preparations.[4] The structure of the side chain thus appeared to have a strong influence on the lanosterol metabolism. The effects of 7-oxo-DHL (**5**),[5] which is a potent inhibitor of sterol synthesis on the metabolism of DHL and 27-nor-DHL (**1b**), were also examined in the present experiments.

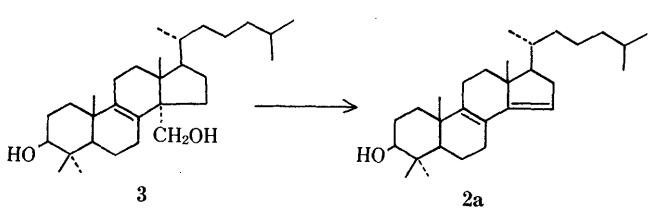

Chart 2

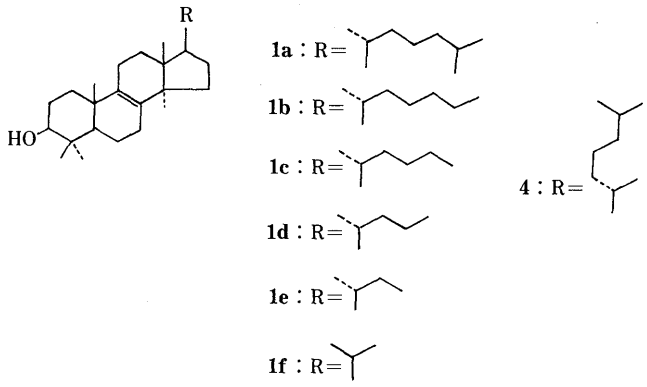

Fig. 1. Structures of the Substrates (**1a**—**f** and **4**)

Experimental Procedures

Materials 24,25-Dihydrolanosterol analogs (**1b—f**) and 20-iso-DHL (**4**) were synthesized as described previously.[6] 4,4-Dimethyl-5α-cholesta-8,14-dien-3β-ol (**2a**) was synthesized by the method of Paik *et al.*[7] DHL (**1a**) was prepared by hydrogenation of a commercial mixture of lanosterol and 24,25-dihydrolanosterol. 7-Oxo-DHL (**5**) was prepared by the method of Pinky *et al.*[8] Dilauroyl phosphatidyl choline (DLPC) was obtained from Sigma Chemical Co. Other chemicals and biochemicals used were of the highest quality available commercially.

Preparation of Partially Purified Cytochrome P-450$_{14DM}$ The procedures adopted followed the method of Iwasaki *et al.*[9] Microsomes were prepared from livers of male Wistar rats (220—230 g). The 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 P-450 fraction eluted with 10 mM potassium phosphate buffer (pH 7.4) (KPB) containing 0.2% Emulgen 913 was subjected to DE-52 column chromatography at room temperature with a linear gradient of NaCl (0—180 mM), and the P-450 fraction eluted first was subjected to hydroxyapatite column chromatography. The P-450 fraction eluted with 200 mM KPB was collected and the Emulgen 913 was removed by stirring with Biobeads. The remaining Emulgen 913 amounted to *ca.* 0.01%. The specific content of the final enzyme was 4.12 nmol/mg protein. The partially purified cytochrome P-450$_{14DM}$ was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The major band of P-450 was found to have an apparent molecular weight of 51000,[10] and was accompanied with small amounts of some other bands. Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Preparation of Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)–Cytochrome P-450 Reductase This enzyme was purified to a specific activity of 10000 units/mg protein from rat liver microsomes according to the method of Yasukochi and Masters.[12] The enzyme so prepared revealed only one band on SDS-PAGE.

Assay for 14-Demethylation by the Reconstituted System The substrate was dispersed with DLPC as described by Aoyama *et al.*[1] 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 substrate solution dispersed with DLPC were mixed and sonicated, and 0.36 ml of 0.1 M KPB (pH 7.4) consisting of 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 the mixture was incubated 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, and 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 evaporating 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 the 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 temperature were 270, 255, and 260 °C, respectively. The lowest limit of detection by mass chromatography was *ca.* 5 ng. On the other hand, the extracted sterols from the reaction mixture were analyzed by high-performance liquid chromatography (HPLC), which was performed on a μBondapak C_{18} reverse-phase column (3.9 mm × 30 cm), uisng 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 employed as the eluent (flow rate, 1.0 ml/min). For 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 peaks of ergosterol and the metabolite.

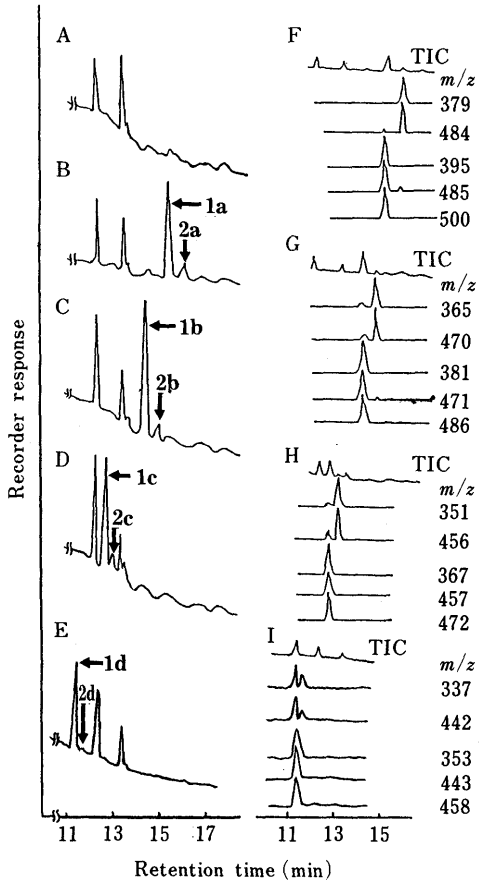

Fig. 2. Gas Chromatographic Detection of Metabolites of DHL, 27-Nor-DHL, 26,27-Dinor-DHL, and 25,26,27-Trinor-DHL and Their Mass Chromatograms

Panel A: Incubation was carried out without substrate as described below. Extract from the reaction mixture was analyzed as described below.

Panel B: DHL (23 nmol) dispersed with DLPC (50 μg) was incubated with the reconstituted system consisting of 0.18 nmol partially purified cytochrome P-450$_{14DM}$ and 1 unit of NADPH–cytochrome P-450 reductase. The reaction mixture was incubated at 37 °C for 30 min on air. Sterols from the reaction mixture were trimethylsilylated and analyzed by gas chromatography–mass spectrometry with an SP-2250 capillary column. Peaks **1a** and **2a** represent trimethylsilylated derivatives of DHL and the 14-demethylated product, respectively.

Panel C: 27-Nor-DHL (23 nmol) dispersed with DLPC (50 μg) was incubated and the sterols in the reaction mixture were analyzed as above. Peaks **1b** and **2b** represent trimethylsilylated derivatives of 27-nor-DHL and the 14-demethylated product, respectively.

Panel D: 26,27-Dinor-DHL (23 nmol) dispersed with DLPC (50 μg) was incubated and the sterols in the reaction mixture were analyzed as described in Panel B. Peaks **1c** and **2c** represent trimethylsilylated derivatives of 26,27-dinor-DHL and the 14-demethylated product, respectively.

Panel E: 25,26,27-Trinor-DHL (23 nmol) dispersed with DLPC (50 μg) was incubated and the sterols in the reaction mixture were analyzed as described in Panel B. Peaks **1d** and **2d** represent trimethylsilylated derivatives of 25,26,27-trinor-DHL and the 14-demethylated product, respectively.

Panel F: 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 by measuring the total ion current.

Panel G: Mass chromatogram of the product in Panel C. The column effluent was monitored by mass chromatography at *m/z* 365, 470, 381, 471, and 486, as well as by measuring the total ion current.

Panel H: Mass chromatogram of the product in Panel D. The column effluent was monitored by mass chromatography at *m/z* 351, 456, 367, 457, and 472, as well as by measuring the total ion current.

Panel I: Mass chromatogram of the product in Panel E. The column effluent was monitored by mass chromatography at *m/z* 337, 442, 353, 443, and 458, as well as by measuring the total ion current.

Results and Discussion

Metabolism of 24,25-Dihydrolanosterol Analogs by the Reconstituted System The partially purified cytochrome P-450$_{14DM}$ with a specific content of 4.12 nmol/mg protein was employed in the present investigation. The metabolism of **1a—f** and **4** was studied using a reconstituted system consisting of partially purified cytochrome P-450$_{14DM}$ and NADPH–cytochrome P-450 reductase. Among the above compounds, **1a—d** were metabolized but the others (**1e,f**, and **4**) were not. Figure 2A shows a gas-chromatogram (GC) of the extract of a reaction without substrate. Figure

2B illustrates the gas-chromatographic detection of DHL (**1a**) and its metabolite (**2a**). The metabolite, which had a relative retention time (Rt_R) of 1.04 with respect to **1a**, was identified as 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**2a**) by comparison with an authentic sample, and this compound was further proved to be the only metabolite formed from the substrate by the reconstituted system under such conditions. Figures 2C, 2D and 2E illustrate the gas chromatographic detection of other substrates (**1b—d**) and their metabolites (**2b—d**). It is clear from the chromatograms that **1b** and **1c** were converted to the corresponding metabolites. Each metabolite (**2b** and **2c**) revealed an Rt_R of 1.04 with respect to each substrate. However, in the case of **1d** (Fig. 2E), the formation of the metabolite (**2d**) was not clear on GC analysis. The mass chromatogram (Fig. 2I) demonstrated the formation of **2d**. Figure 2F shows a mass chromatogram of **1a** and its metabolite (**2a**). 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 (**2a**), respectively. The ions at m/z 500, 485 and 395 can be identified as the molecular ion (M^+), $M^+ - CH_3$ and $M^+ - CH_3 - TMSOH$ of **1a**, respectively. Figure 2G presents a mass chromatogram of **1b** and its metabolite (**2b**). The ions at m/z 470 and 365 can be identified as M^+ and $M^+ - CH_3 - TMSOH$ of the 14-demethylated product (**2b**), respectively. The ions at m/z 486, 471 and 381 can be identified as M^+, $M^+ - CH_3$ and $M^+ - CH_3 - TMSOH$ of **1b**, respectively. Figures 2H and 2I present mass chromatograms of **1c** and **1d** and their metabolites (**2c**, **d**). These ions can be identified as described for Figs. 2F and 2G. Figure 3 shows mass spectra of the metabolites (**2a—d**). A comparison of the mass spectra and Rt_R values indicated that the metabolites were the 14-demethylated products. Following a 60 min incubation, the conversion ratios of **1a—d** were calculated from the peak areas of the substrates and the metabolites to be about 17, 10, 9, and 2%, respectively. Under the same conditions, the conversion ratio of lanosterol was estimated to be 19% (data not shown).

The metabolites generated by the reconstituted system could also be detected by HPLC assay as illustrated in Fig. 4. The peaks in Figs. 4B, C, D and E show the sterols in the extracts of the reaction mixtures of DHL (**1a**), 27-nor-DHL (**1b**), 26,27-dinor-DHL (**1c**) and 25,26,27-trinor-DHL (**1d**), respectively. Figure 4A depicts the extract of the reaction mixture without substrate. The detection at 248 nm presented unambiguous evidence for the presence of the 8,14-diene systems. Each metabolite (**2b**, **2c** and **2d**) revealed an Rt_R of 0.72 with respect to each substrate (**1b**, **1c** and **1d**). This Rt_R coincided with that of the metabolite (**2a**) to DHL (**1a**). The metabolites are therefore inferred to be 27-nor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-

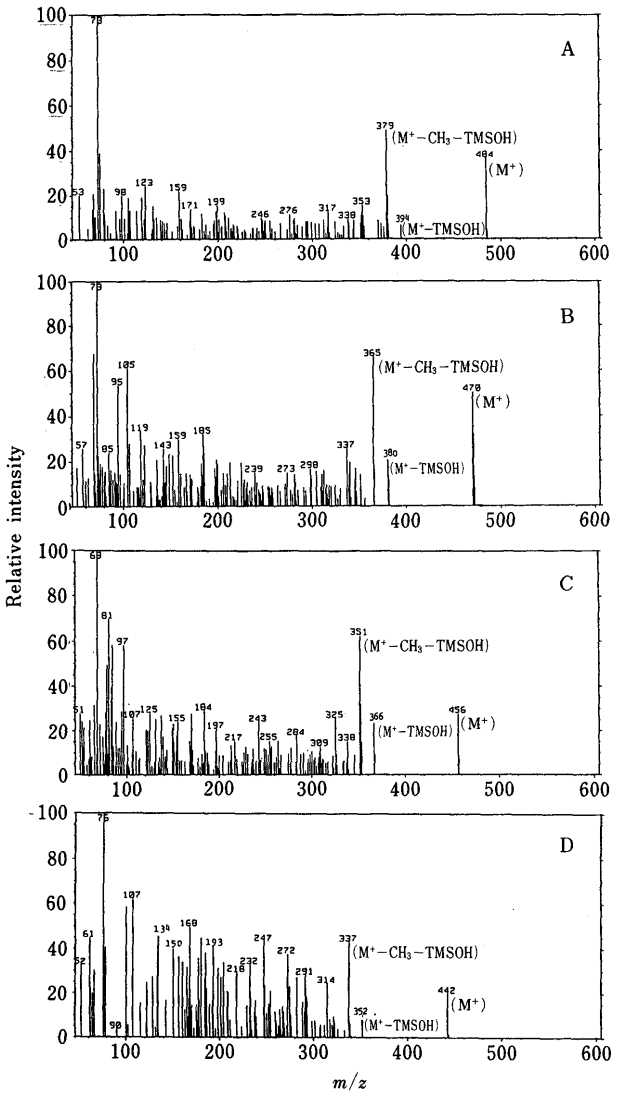

Fig. 3. Mass Spectra of the Metabolites (**2a**, **2b**, **2c** and **2d**)

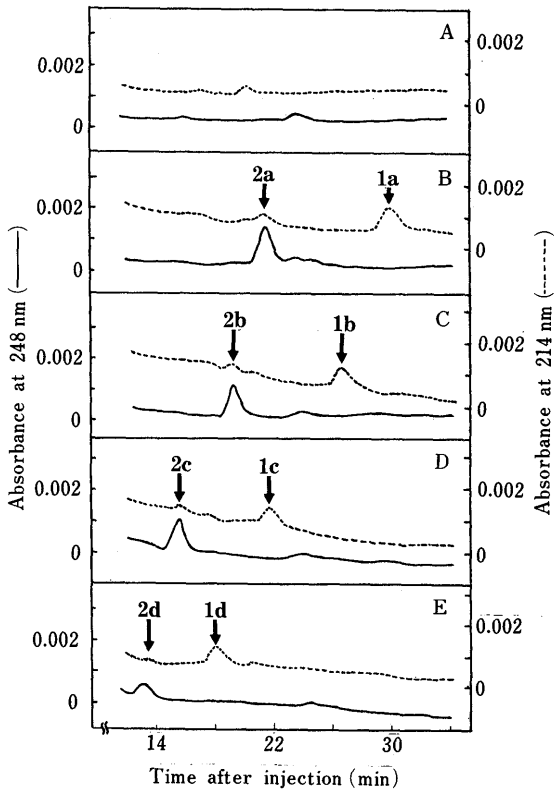

Fig. 4. Reverse-Phase HPLC Chromatographic Detections at 214 and 248 nm of Metabolites from DHL, 27-Nor-DHL, 26,27-Dinor-DHL and 25,26,27-Trinor-DHL by the Reconstituted System

Peaks **1a—d** and **2a—d** correspond to the substrates and their metabolites.
Panel A: Sterols in the extract in the reaction without substrate.
Panel B: Sterols in the extract in the reaction of DHL.
Panel C: Sterols in the extract in the reaction of 27-nor-DHL.
Panel D: Sterols in the extract in the reaction of 26,27-dinor-DHL.
Panel E: Sterols in the extract in the reaction of 25,26,27-trinor-DHL.

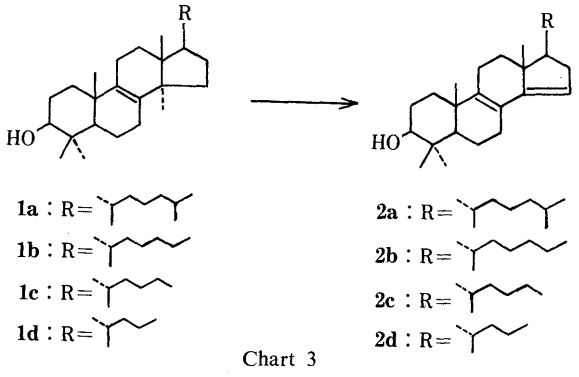

Chart 3

ol (**2b**), 26,27-dinor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**2c**), and 25,26,27-trinor-4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (**2d**). It can be concluded that the reconstituted system catalyzed the removal of the 14-methyl group of 27-nor-, 26,27-dinor-, and 25,26,27-trinor-DHL as well as DHL, forming the corresponding 8,14-diene compounds (Chart 3).

As shown in Fig. 5, the apparent K_m value of the reconstituted system for DHL was determined as 19.2 μM while those for 27-nor-DHL and 26,27-dinor-DHL were 38.8 and 41.7 μM, respectively. Further, the V_{max} values for **1a**—**c** determined from Fig. 5 were 0.22, 0.19 and 0.18 nmol/min/nmol cytochrome P-450_{14DM}, respectively. These results indicate that cytochrome P-450_{14DM} has a higher affinity for DHL than for the 27-nor and 26,27-dinor analogs (**1b** and **1c**) with shorter side chains. Thus, the structure of the side chain may strongly influences the rates of 14-demethylation of lanosterol analogs by cytochrome P-450_{14DM}, as suggested for the metabolism of lanosterol analogs by rat liver homogenate preparations.[3,4] Since 14-demethylation is the essential step in cholesterol biosynthesis, the participation of other P-450s could be neglected, although the cytochrome P-450_{14DM} used was the partially purified enzyme. The present results indicate that the substrate specificity of cytochrome P-450_{14DM} for steroids with different lengths of side chain is lower than that for the double bond positions on the steroid nucleus.

Yeast cytochrome P-450 which catalyzes lanosterol 14α-demethylation, has been purified by Yoshida and Aoyama.[13] Also, Trzaskos *et al.*[10] recently described the purification of lanosterol 14α-methyl demethylase cytochrome P-450 (specific content, 15.8 nmol/mg protein) from rat hepatic microsomes.

Effect of 7-Oxo-DHL on the Metabolism of DHL and 27-Nor-DHL by the Reconstituted System We have demonstrated that 7-oxo-DHL (**5**) is a potent inhibitor[5,14] of lanosterol or DHL metabolism and shows hypolipidemic effects following administration[15] in the diet to male Wistar rats. Further, we have shown that 7-oxo-DHL is the first example of a cytochrome P-450 inhibitor which selectively interferes with the electron transfer to the oxyferro intermediate of cytochrome P-450_{14DM} of *Saccharomyces cerevisiae*.[16] In the present study, it was found that **5** inhibited the demethylation of the 14-methyl group from DHL (**1a**) by the reconstituted system, as shown in Fig. 6. Similarly, when 27-nor-DHL (**1b**) was used as a substrate, **5** inhibited the demethylation. These results suggest that the side chain analogs, **1a** and **1b**, are almost the same as the substrate of cytochrome P-450_{14DM}, indicating that the

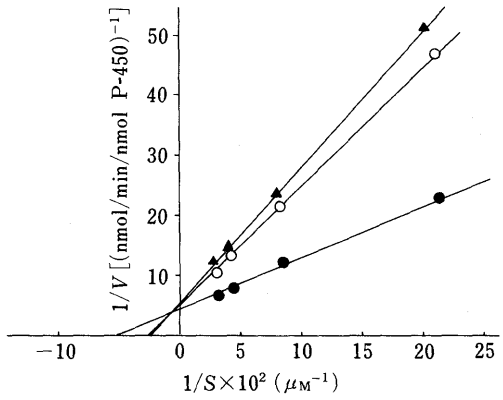

Fig. 5. Double Reciprocal Plots of DHL, 27-Nor-DHL and 26,27-Dinor-DHL Metabolism by the Reconstituted System

The metabolism of DHL, 27-nor-DHL, and 26,27-dinor-DHL was assayed as described in Fig. 2 except that the concentration of the substrates was varied as indicated. ●, DHL; ○, 27-nor-DHL; ▲, 26,27-dinor-DHL.

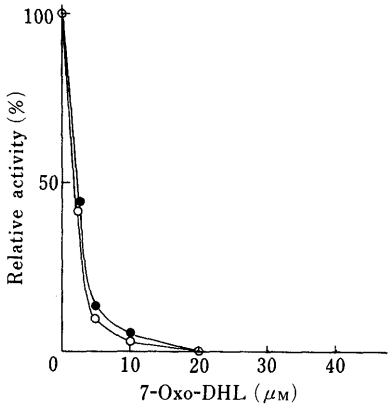

Fig. 6. Effects of 7-Oxo-DHL on the Metabolism of DHL and 27-Nor-DHL by the Reconstituted System

The metabolism of DHL and 27-nor-DHL was assayed as described in Fig. 2 in the presence of the indicated concentrations of 7-oxo-DHL. 7-Oxo-DHL was added to the reaction mixture as a DLPC solution (5 μl). The corresponding volume of solvent was added in the control experiment. ●, DHL; ○, 27-nor-DHL.

removal of the 14-methyl group of **1a** and **1b** is mediated at the same site on cytochrome P-450_{14DM}.

Recently, Frye and Robinson[17] reported that a series of novel 32-functionalized lanost-7-en-3β-ols acted as powerful inhibitors of lanosterol 14α-methyl demethylase in rat liver microsomes using a modification of an assay developed by Gaylor *et al.*[18] On the other hand, Aoyama *et al.*[19] purified a cytochrome P-450 (P-450_{SGI}) from a lanosterol 14α-demethylase (P-450_{14DM})-defective mutant of *Saccharomyces*, and demonstrated that P-450_{SGI} was an altered P-450_{14DM}. Further, Chen *et al.*[20] observed that a Chinese hamster ovary cell mutant was lanosterol 14α-methyl demethylase-deficient.

The present results provide useful information concerning the substrate specificity of cytochrome P-450_{14DM}, especially in relation to the contribution made by the structure of the sterol side chain.

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