Lanosterol 14α-demethylase ($P450_{14DM}$): effects of $P450_{14DM}$ inhibitors on sterol biosynthesis downstream of lanosterol

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Abstract Lanosterol 14α-demethylase ($P450_{14DM}$) is the cytochrome P450 enzyme complex responsible for an early step in cholesterol biosynthesis, namely the 14α-demethylation of lanosterol. We have synthesized a novel series of steroidal substrate analogues, designed to be specific and potent inhibitors of $P450_{14DM}$. We describe here the effects of these compounds on sterol biosynthesis downstream from lanosterol, focusing ultimately on their efficacy as inhibitors of cholesterol biosynthesis. Results using a radio-high performance liquid chromatography (HPLC) assay show that in rat liver microsomal preparations, with [24, 25-^{3}H]dihydrolanosterol as substrate, the compounds do indeed inhibit the biosynthesis of sterols downstream from lanosterol. A range of inhibitory potencies was observed, and the key enzyme being inhibited was believed to be $P450_{14DM}$. Inhibitor efficacy was readily correlated with non-metabolized [24,25-^{3}H]dihydrolanosterol, formation of 4,4-dimethyl-cholest-8-en-3β-ol, and formation of lathosterol, a sterol believed to be an excellent indicator of whole body cholesterol biosynthesis in humans. —**Tuck, S. F., H. Patel, E. Safi, and C. H. Robinson.** Lanosterol 14α-demethylase ($P450_{14DM}$): effects of $P450_{14DM}$ inhibitors on sterol biosynthesis downstream of lanosterol. *J. Lipid Res.* 1991. **32:** 893–902.

Supplementary key words lanosterol • cholesterol • lathosterol • radio-HPLC assay

Cholesterol 1 is ubiquitous in humans; it is both an essential component of cell membranes and a precursor for the steroid hormones and bile acids. It is also essential for the growth and function of yeasts and fungi. Two sources of cholesterol exist for the mammalian system: exogenous (i.e., dietary) sterols and endogenous sterols biosynthesized from acetyl-CoA. Disorders of cholesterol biosynthesis and regulation can result in elevated serum cholesterol levels and atherosclerosis.

Lanosterol 14α-demethylase ($P450_{14DM}$) is the cytochrome P450 enzyme complex responsible for an early step in cholesterol biosynthesis, namely the 14α-demethylation of lanosterol 2 (1) (**Scheme 1**). The 14α-methyl group is removed via three oxidative steps, each requiring one equivalent of NADPH and molecular oxygen. The overall process results in the loss of the methyl group as formic acid and formation of the 8,14-diene 3 (2) (Scheme 1). Inhibitors of $P450_{14DM}$ are not only of interest as mechanistic probes of the enzyme, but also as potential therapeutic agents for treatment of hypercholesterolemia and as antimycotics.

We have synthesized a series of steroidal substrate analogues for $P450_{14DM}$, designed to be specific and potent inhibitors of this enzyme. We describe here the effects of these compounds on sterol biosynthesis downstream from lanosterol, focusing ultimately on their efficacy as inhibitors of cholesterol biosynthesis. Results, using a radio-HPLC assay first described by Trzaskos et al. (1), show that for microsomal liver preparation with [24, 25-^{3}H]dihydrolanosterol as substrate, our compounds do indeed inhibit the biosynthesis of sterols downstream from lanosterol. A range of inhibitory potencies was observed, and the key enzyme being inhibited was believed to be $P450_{14DM}$. Inhibitor efficacy was readily correlated with non-metabolized [24,25-^{3}H]dihydrolanosterol, formation of 4,4-dimethyl-cholest-8(9)-en-3β-ol 4, and formation of lathosterol 5, a sterol believed to be an excellent indicator of whole body cholesterol biosynthesis in humans (3).

MATERIALS AND METHODS

Materials

Cholesterol, ketoconazole, DL-isocitric acid, trisodium salt; β-NADP, sodium salt; β-NADPH tetrasodium salt,

Abbreviations: HPLC, high performance liquid chromatography; THF, tetrahydrofuran; DHL, dihydrolanosterol; PGE, phosphate-glutathione-EDTA; EI, electron impact; TLC, thin-layer chromatography; HRMS, high resolution mass spectrometry.

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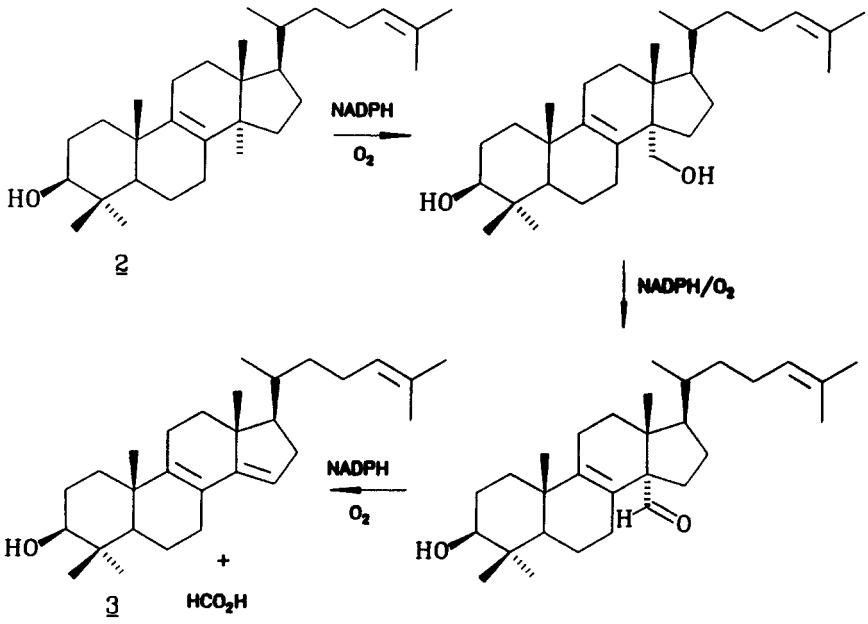
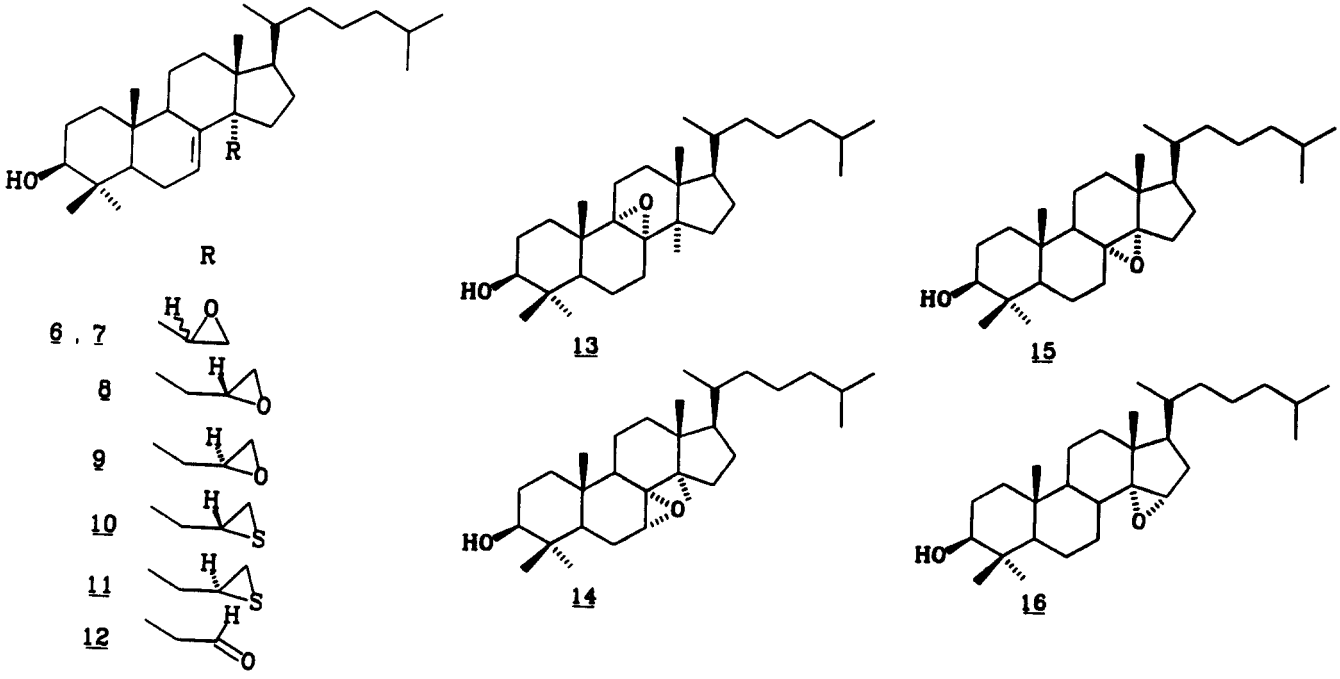


Scheme 1. Lanosterol 14α-demethylase pathway.

type I; β-NAD, grade III; β-NADH, disodium salt, grade III; isocitric dehydrogenase, type IV, porcine heart; and Tyloxapol were purchased from Sigma Chemical Co., St. Louis, MO. AY9944 was a gift from Ayerst, Philadelphia, PA, and analytical grade NaCN was from Mallinckrodt Inc., St. Louis, MO. Lathosterol and 24,25-dihydrolanost-7-en-3β-ol were synthesized by standard procedures. [7-^{3}H(N)]Cholesterol (21 Ci/mmol) was purchased from NEN Products (Boston, MA).

Synthesis of inhibitors

Sterols 8–12 (**Scheme 2**) were synthesized as described previously (4). Epoxysterols 13 and 14 (Scheme 2) were prepared as previously described (5).



Scheme 2.

4,4-Dimethyl-8(14)α-epoxycholestan-3β-ol (15) and 4,4-dimethyl-14α,15α-epoxycholestan-3β-ol (16)

4,4-Dimethyl-cholest-8(14)-en-3β-ol (300 mg, 0.72 mmol) was dissolved in chloroform (10 ml) at room temperature and hydrogen chloride gas was passed through the solution for 2 h. The reaction was concentrated in vacuo to give an off-white residue, shown by ^{1}H NMR spectroscopy to be a 60:40 mixture of 4,4-dimethyl-cholest-8(14)-en-3β-ol and 4,4-dimethyl-cholest-14-en-3β-ol. The crude mixture was dissolved in dichloromethane (10 ml), and sodium bicarbonate (120 mg, 1.45 mmol) and *m*-chloroperoxybenzoic acid (MCPBA) (22 mg, 1.4 mmol) were added. TLC indicated that the reaction was complete after 15 min. The reaction mixture was washed twice with 5% aqueous sodium hydroxide solution, twice with water, and the organic phase was dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (EtOAC–hexanes 1:9) followed by reverse phase HPLC (octyl, H_2O–MeOH 1:9) to give the two pure products: 15 (85 mg, 24%, 0.20 mmol): mp 112°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.24 (dd, 14.0 Hz 1.1 Hz, 1, H-3); MS *m/z* 430 (M^+); HRMS, Calcd 430.3811 ($C_{29}H_{50}O_2$), Found 430.3799; Anal. Calcd ($C_{29}H_{50}O_2$) C, 80.86 H, 11.71: Found C, 80.78 H, 11.74 and 16 (101 mg, 35%): mp 164°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (dd, 14.3 Hz 1.1 Hz, 1, H-3) 3.33 (s, 1, H-15); MS *m/z* 430 (M^+); HRMS, Calcd 430.3811 ($C_{29}H_{50}O_2$), Found 430.3801; Anal. Calcd ($C_{29}H_{50}O_2$) C, 80.86; H, 11.71: Found C, 80.91; H, 11.77.

4,4-Dimethyl-14α-oxiranyl-cholest-7-ene-3β-ols (6 and 7)

In a 10-ml round-bottom flask, under argon, was placed 50% oil-dispersed sodium hydride (26 mg, 0.54 mmol). This dispersion was washed twice with dry tetrahydrofuran (THF) (1 ml), and the solvent was removed under argon. Dry dimethylsulfoxide (DMSO, 0.4 ml) was added and the mixture was heated to 70–75°C under argon, with stirring, for 30 min. The reaction mixture was cooled to 25°C, dry THF (1.0 ml) was added, and the mixture was cooled to 0°C. A solution of trimethylsulfonium iodide (140 mg, 0.69 mmol) in dry DMSO (0.5 ml) was added dropwise with stirring. After the addition was complete, stirring was continued, and after 5 min at 0°C a solution of 32-oxo-lanost-7-en-3β-ol (4) (31 mg, 0.07 mmol) in dry THF (0.3 ml) was added dropwise. The resulting mixture was stirred at 0°C for 30 min followed by 6 h at room temperature under argon. The reaction mixture was concentrated in vacuo, the residue was taken up in dichloromethane, and the solution was washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The residue was purified by chromatography on silica gel (EtOAC–hexanes 1:4) to give two products. Further purification by normal phase HPLC (Whatman Magnum 10 Partisil, EtOAC–hexanes 1:4) gave the two pure products: 6 (12 mg, 0.025 mmol, 35%): mp 105–106°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.52 (m, 1, epoxide H), 2.62 (t, J = 4.0 Hz, 1, epoxide-H), 3.24 (m, 2, H-32 H-3), 5.18 (m, 1, H-7); MS *m/z* 456 (M^+); HRMS, Calcd 456.3967 ($C_{32}H_{50}O_2$), Found 456.3974; Anal Calcd ($C_{32}H_{50}O_2$) C, 84.14; H, 11.48: Found C, 84.01; H, 11.43; and 7 (12 mg, 0.025 mmol, 35%): mp 110–111°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.30 (dd, J = 4.8 Hz 2.8 Hz, 1, epoxide-H) 2.60 (dd, J = 4.4 Hz 4.0 Hz, 1, epoxide-H), 3.24 (m, 1, H-3), 3.30 (dd, J = 3.6 Hz 3.2 Hz, 1, H-32), 5.18 (m, 1, H-7); MS *m/z* 456 (M^+); HRMS, Calcd 456.3967 ($C_{32}H_{50}O_2$), Found 456.3969; Anal. Calcd ($C_{32}H_{50}O_2$) C, 84.14; H, 11.48: Found C, 84.31; H, 11.50.

Isolation of microsomes

Microsomes were prepared using the method of Trzaskos et al. (1) Male Sprague-Dawley rats were maintained on a diet of regular rat chow and killed by asphyxiation with CO_2. Protein concentrations were determined using the Bradford assay (6), with bovine serum albumin as a standard.

Preparation of [24,25-^{3}H]dihydrolanosterol

Lanosterol was isolated from a commercial mixture of 24,25-dihydrolanosterol (24,25-DHL) and lanosterol (Sigma Chemical Co.) by reverse phase HPLC using an Ultrasphere Octyl Column (5 μM, 9.6 mm × 25 cm), methanol as the eluant, and RI detection. [24,25-^{3}H]Dihydrolanosterol (137 Ci/mmol) was purchased from Amersham International PLC. Water-soluble counts (<1%) were removed by repeated water extraction of a solution of the steroid in dichloromethane. The radiolabeled steroid was stored at −20°C in ethanol and purified prior to use, using HPLC System I as described in Assay conditions. Both the [24,25-^{3}H]DHL and the nonradiolabeled 24,25-DHL displayed a single peak when chromatographed using HPLC System I (^{3}H and RI detection). Typically, purity was greater than 99%. For the unlabeled 24,25-DHL used to dilute the tritiated material, electron impact mass spectroscopy showed m/e 428 (M^+). Similarly 90 MHz NMR spectroscopy showed δ = 3.29 (m, 1H, H-3) and 5.20 (m, 1H, H-7), which are characteristic resonances for 24,25-DHL.

Assay conditions

Substrate [24,25-^{3}H]dihydrolanosterol (47.6 mCi/mmol) was dissolved in 0.1 M KH_2PO_4 (pH 7.4) with the aid of Tyloxapol in the ratio of 75:1 (w/w, detergent to steroid). The incubation contained, in a final volume of 1.0 ml, 0.1 M KH_2PO_4 buffer (also with 1 mM glutathione, 0.1 mM EDTA, pH 7.4; PGE buffer), 2 mg microsomal protein, 0.2 mM NAD^+, 0.1 mM NADH, 2.0 mM NADPH,

0.3 mM $NADP^+$, 10 mM isocitrate, 0.25 units of isocitric dehydrogenase, 0.4 mM $MgCl_2$, and inhibitors at various concentrations. Incubations were started by addition of steroid substrate (18 μM, 47.6 mCi/mmol, 1.9×10^6 dpm) and continued at 37°C for 3 h. Incubations were terminated by addition of 1 ml 15% KOH in 95% MeOH, and the samples were heated under a stream of air in a water bath at 90°C for 30 min and steroids were then extracted with 4 × 2 ml petroleum ether. The combined fractions were evaporated under air at 50°C and the residue was dissolved in 1 ml EtOH of which 100 μl was used to determine the percent recovery (Beckman LS7000 scintillation counter). Conversion of [24,25-^{3}H]DHL to products was assessed by reverse phase HPLC. Thus, 100 μl was injected on a 5-μm octyl column (4.6 mm × 2.5 cm) equipped with a 1-cm pre-column, and chromatography was performed at 25°C and 1.5 ml/min using MeCN–MeOH–H_2O 45:45:10 (HPLC System I). A second eluant system of 1.5 ml/min and MeCN–MeOH–H_2O 42.5:42.5:15 (HPLC System II) was used for the chromatography when cholesterol and lathosterol were required to be separated and quantified. Steroids were detected using a Flo-one Beta detector (Model CR) (2.5 ml flow cell for HPLC System I, update time 6 sec; 0.5 ml flow cell for HPLC System II, update time 2 sec) using Flo-Scint II as scintillant (scintillant volume: column effluent volume = 2:1). Under these conditions, a static counting efficiency of 30% was obtained for tritium. [24,25-^{3}H]DHL metabolism and product yields were quantified using the Radiomatics High Level Integration Program, set in the DPM mode with the background subtract of 150 dpm and a minimum peak area of 500 dpm.

Inhibitors

All steroids used in inhibitor studies were analytically pure. Stock solutions of the individual compounds were prepared on the day of use. Typically, the steroid (100 μg–2 mg) was dissolved in benzene to give a concentration of 2.5 mM, and this stock solution was used to prepare compounds for assays. The desired stock solution (200 μl) was mixed with 75 μl of a solution of Tyloxapol (31 mg) in acetone (1 ml) producing a detergent to steroid ratio of 75:1. This solution was evaporated under a stream of nitrogen and the residue was dissolved in PGE buffer (68 μl) to produce a final sterol concentration of 1.0 mM. Aliquots of this solution were used directly in the assay. Solutions of AY9944, sodium cyanide, and ketoconazole were prepared in a similar manner, with the exception of NaCN which was dissolved directly in the PGE buffer in the presence of detergent. For all assays a fixed concentration of detergent was used, determined by a 75:1 ratio to the highest inhibitor concentration. This was necessary to overcome a noncompetitive inhibitory effect of the detergent on $P450_{14DM}$ observed by us and by others (7). For assays performed in the absence of inhibitor, the detergent concentration was maintained at the same level as in the presence of inhibitor.

Biosynthesis of 4,4-dimethyl-cholest-8(9)-en-3β-ol (4)

Sterol 4 was biosynthesized by a procedure similar to that described by Shafiee et al. (8) with the following modifications. A 250-ml Erlenmeyer flask was found to be the most suitable reaction vessel, as this allowed for sufficient aeration of the reaction solution. Use of a smaller vessel, with a smaller reaction surface area available to the atmosphere, decreased the product yield dramatically. Purification was performed by reverse phase HPLC, using HPLC System I as described in Assay conditions and with RI detection. The product with a relative retention time of 0.90 was collected. Mass spectral (EI) and high field NMR analysis (400 MHz) confirmed this compound to be the desired 14α-demethylated sterol.

Isolation and identification of cholesterol and lathosterol

Cholesterol and lathosterol were biosynthesized as described in Assay conditions. Subsequent isolation and purification was by reverse phase HPLC (HPLC System II). The authenticity of the steroids isolated in this manner was established by comparison of retention times with authentic material and co-crystallization with authentic carrier. (See Identification of metabolites.)

RESULTS

Development of assay conditions

Initially, we established incubation times and substrate concentrations that would permit the use of the radio-HPLC assay for examining the effects of our inhibitors on sterol biosynthesis from [24,25-^{3}H]dihydrolanosterol ([24,25-^{3}H]DHL) in the microsomal fraction of rat liver homogenate. In particular, we wished not only to chromatographically resolve important metabolites that could act as markers for sterol biosynthesis, but to measure cholesterol, so as to examine the effects of our compounds on cholesterol biosynthesis. It was necessary to determine conditions that would produce sufficient yields of metabolites for accurate quantification by digital integration of the radio-HPLC peak areas. Incubations of 18 μM and 36 μM [24,25-^{3}H]DHL were performed for time increments up to 5 h in the presence of 2 mg of microsomal protein to determine optimum incubation time. Chromatographic examination of the radio-HPLC assays using both HPLC Systems I and II demonstrated that cholesterol and lathosterol are not resolved by System I, but are fully resolved by System II.

TABLE 1. Cholesterol production[a] by incubations of [24,25-^{3}H]DHL with four different preparations of rat liver microsomes

Microsomes[b]	Lathosterol 5/dpm	Cholesterol 1/dpm	% Cholesterol
I	10,054	418	4.0
II	8,457	481	5.4
III	9,629	1,618	14.4
IV	8,120	300	3.6

Incubations and assays were performed as described in Assay conditions. HPLC system II was used for metabolic analysis.

[a]As a function of total combined lathosterol and cholesterol.

[b]All four batches of microsomes were prepared using the same procedure and stored in a similar manner.

Results from assays with four different batches of microsomes (**Table 1**) show that the lathosterol to cholesterol ratio varies over a narrow range, with cholesterol accounting for an average of only 7% of these two C-27 sterols. Further, no cholesta-5,7-dien-3β-ol (the intermediate through which cholesterol is synthesized from lathosterol) was detected in any of our assays (HPLC System I R_f 0.57). This suggests that, under the incubation conditions used here, the Δ^7-sterol 5-desaturase is possibly the rate-determining enzyme in the later stages of cholesterol biosynthesis (**Scheme 3**), in agreement with the postulate of Shafiee et al. (8). One might expect lathosterol to be exclusively metabolized to cholesterol; hence we believe that combined lathosterol and cholesterol yields are a suitable indicator of potential cholesterol biosynthesis and report here values obtained by digital integration of peak areas obtained using HPLC System I.

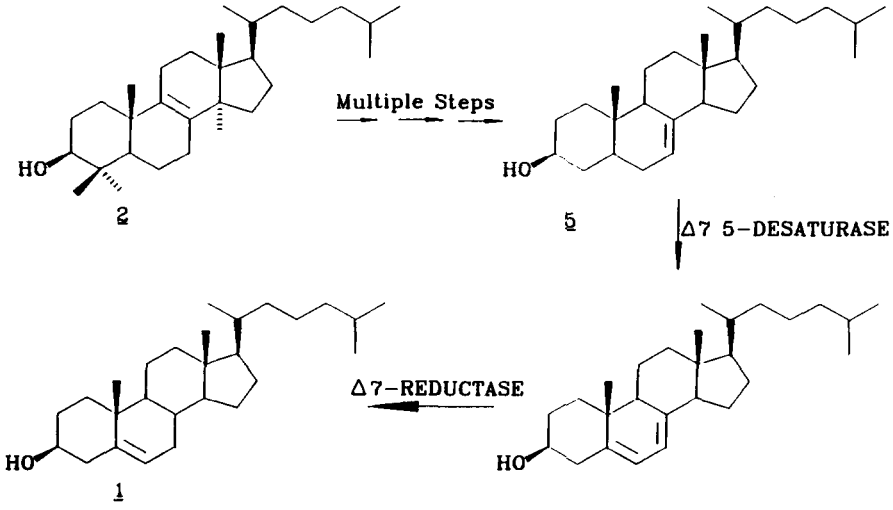
The results plotted in **Fig. 1** suggested optimum incubation conditions of 3 h at 37°C. After 3 h, approximately 67% [24,25-^{3}H]DHL had been metabolized, resulting in the accumulation of approximately 40% of 14α-demethylated metabolite 4 and 7% (combined total) of lathosterol and cholesterol. Furthermore, total lathosterol and cholesterol synthesis appeared to be linear over the time range examined. Typical recovery of radioactivity, as determined by both static scintillation counting of the assay extract and continuous detection in the F1β system, was greater than 85%. **Fig. 2** shows typical chromatograms for control assays, employing both HPLC Systems I and II.

Identification of metabolites

Peak A was identified as [24,25-^{3}H]DHL by comparison of the retention time with authentic material. The possibility of co-elution and contamination with 24,25-unsaturated metabolites is eliminated by the use of 24-25-dihydrolanosterol as substrate. Furthermore, the use of a tritium-labeled substrate and the detection of only tritium-labeled metabolites eliminates interference by detection of endogenous sterols. Comparison of the retention time of an authentic sample of 24,25-dihydrolanost-7-en-3β-ol (R_f 0.93) with that of [24,25-^{3}H]DHL (R_f 1.0) showed no contamination of [24,25-^{3}H]DHL with the Δ^7 isomer. This is in agreement with the observations of Gaylor, Delwiche, and Swindell (9) that 24,25-DHL is not a substrate for the Δ^8-Δ^7 isomerase. Alternatively, failure to detect isomerization may be the result of the position of equilibrium of this reaction favoring the Δ^8 isomer, since Schroepfer and Pascal (10) have observed the ready isomerization of 14α-methyl Δ^7-sterols to their Δ^8 counterparts.

Peak B was identified as 4,4-dimethyl-cholest-8(9)-en-3β-ol (4) (R_f 0.90) by comparison with authentic material, obtained by biosynthesis from 24,25-dihydrolanosterol as described in the Methods Section.

Peaks D and E have been shown previously by Trzaskos et al. (1) and Gaylor et al. (9) to contain 4-monomethyl



Scheme 3.

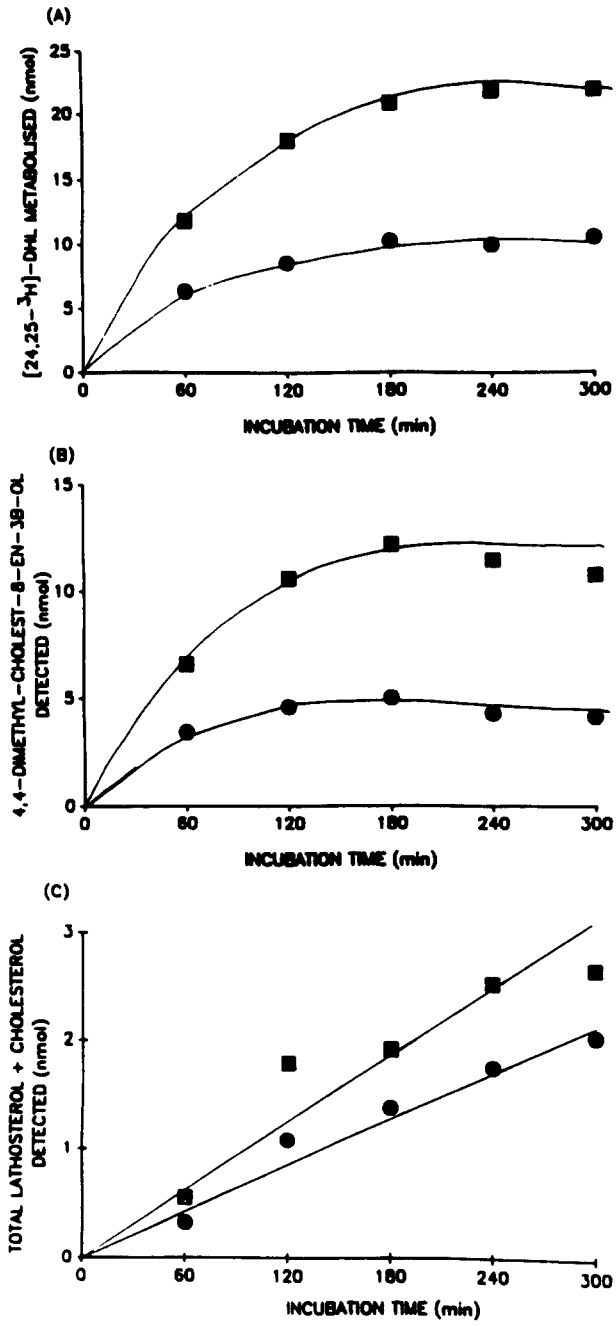


Fig. 1. Linearity of substrate metabolism and metabolite formation. Radio-HPLC assay was performed as described in Assay conditions with 2 mg of protein at 37°C; ■, 36 μM; ●, 18 μM. A: Extent of substrate metabolism. B: Formation of 4,4-dimethyl-cholest-8-en-3β-ol. C: Formation of total lathosterol and cholesterol.

sterols. We have not identified or quantified these sterols in this study as they are directly metabolized to the C-27 sterols lathosterol and cholesterol which are of greater biological interest.

The fraction represented by peak F/G in Fig. 2A contains two separable and isolable C-27 sterols, namely cholesterol 1 and lathosterol 5. This observation was confirmed by comparison of HPLC retention times (HPLC System II) with authentic samples, and by co-crystallization to constant activity (**Table 2**). Lathosterol and cholesterol were separable using HPLC System II. Peak G was isolated by HPLC yielding a sample of 258,320 cpm, and added to 25 mg of lathosterol carrier producing a specific activity of 10,400 cpm/mg. After three recrystallizations from acetone, 95% of the initial radioactivity was associated with the lathosterol (sp act 9850 cpm/mg), confirming the identity of peak G. In a similar fashion, peak F was isolated to yield a sample of 140,700 cpm and added to 25 mg of cholesterol to produce a specific activity of 5630 cpm/mg. After four recrystallizations from ace-

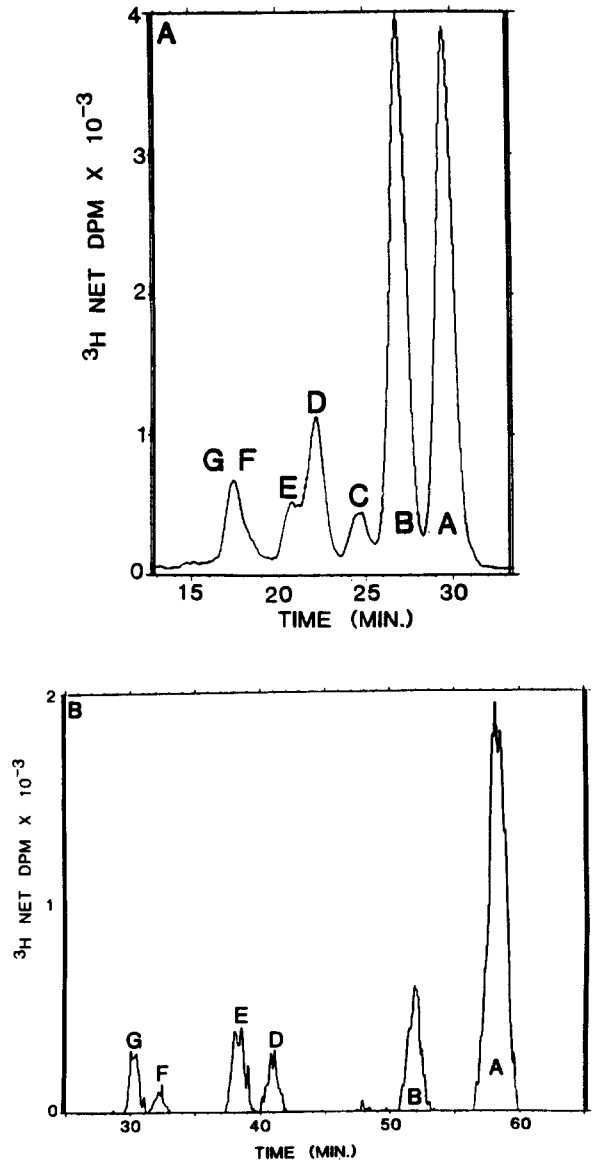


Fig. 2. Radio-HPLC chromatograms of control assays. Assays were performed as described in Assay conditions with 18 μM substrate and 2 mg of microsomal protein for 3 h at 37°C. A: Analysis with HPLC system I. B: Analysis with HPLC system II.

TABLE 2. Crystallization of biosynthesized cholesterol and lathosterol to constant specific activity

Crystallization	Lathosterol[a]		Cholesterol[b]	
	Mother Liquor	Crystals	Mother Liquor	Crystals
	cpm/mg		*cpm/mg*	
1	10100	10050	9600	4630
2	10290	9915	5965	4090
3	10180	9860	4435	4047
4			4121	3901

[a]Original specific activity of lathosterol = 10,400 cpm/mg.
[b]Original specific activity of cholesterol = 5,630 cpm/mg.

tone, 70% of the initial radioactivity was associated with the cholesterol (sp act 3900 cpm/mg), confirming the major component (a minimum of 70%) of peak F to be cholesterol.

Inhibition studies

The compounds shown in Scheme 2 were examined at concentrations of 0, 5, 10, and 20 μM. Their effects on the synthesis of selected metabolites of [24,25-^{3}H]DHL are reported in **Table 3**, as a percentage of the total recovered ^{3}H-labeled sterols.

The sterols with function at C-14 (6–12) all exhibited a dose-dependent inhibitory effect on the biosynthesis of sterols downstream from [24,25-^{3}H]DHL. Compounds 6, 8, and 12, which inhibit total cholesterol and lathosterol biosynthesis by >89% at a concentration of 10 μM, were particularly potent. These three compounds compare extremely well with ketoconazole which produces greater than 90% inhibition at a concentration of 10 μM and exhibits a similar dose-response effect. Indeed, all of the compounds in series 6–12, with the exception of the thiiranes 10, 11, are excellent inhibitors of C-27 sterol biosynthesis.

Analysis of the recoveries of biosynthesized sterol 4 and substrate showed a parallel increase in recovered substrate with the decrease in sterol 4 synthesis, suggesting that compounds 6–12 exert their inhibitory effect, as intended, primarily at the C-14 demethylation step. Furthermore, the decrease in sterol 4 biosynthesis is paralleled by a decrease in the synthesis of sterols downstream from 4, i.e., in the biosynthesis of lathosterol and cholesterol. The potency of the inhibitory effect of sterols 6–12 on C-27 sterol biosynthesis parallels the independently determined K_i values for inhibition of P450$_{14DM}$ (4).

Compounds 13–16, a structurally distinct class from 6–12, all containing nuclear epoxide moieties and no C-14 functionalization, were less potent inhibitors of sterol biosynthesis and did not show the dose-dependent effects of the 6–12 series. The 13–16 series, especially the 8(14)- and 14,15-epoxides, had poorer K_i values for P450$_{14DM}$ inhibition than did sterols 6–12 and this was reflected in their weaker inhibitory effects on the biosynthesis of sterol 4. However, approximately 30% inhibition of total cholesterol and lathosterol biosynthesis was observed for compounds 13, 14, and 16 at a concentration of 10 μM.

In order to put this study into perspective, we also examined three known nonsteroidal inhibitors of specific steps in the biosynthetic pathway from lanosterol to cholesterol. Ketoconazole is an effective anti-fungal drug used in the treatment of systemic mycoses (11), its clinical effect being mediated mainly through inhibition of C14-demethylation of ergosterol (12). AY9944 is a potent inhibitor of the enzymic reduction of the Δ^{14} double bond (12) of 4,4-dimethyl-cholest-8,14-dien-3β-ol 3, the Δ^8–Δ^7 isomerase (13), and the 7,8-double bond of 5,7-diene sterols. The cyanide ion is an inhibitor of 4-methylsterol oxidase. The inhibitory effects of these compounds are also reported in Table 3. Under the specific incubation conditions used here, ketoconazole (7), AY9944 (1), and cyanide (14) all appear to be exerting the previously described effects on sterol biosynthesis.

DISCUSSION

We have studied the effects of a series of sterols, designed as inhibitors of P450$_{14DM}$, on cholesterol biosynthesis in rat liver microsomes. Radio-HPLC chromatographic analysis of selected metabolites generated from [24,25-^{3}H]DHL demonstrated the most probable enzymatic steps blocked by individual inhibitors. Furthermore, we have examined the potencies of these compounds as inhibitors of C-27 sterol biosynthesis (Table 3). Specifically, we were able to monitor three important metabolites: *i*) 4,4-dimethyl-cholest-8(9)-en-3β-ol 4, the product of 14α-demethylation of 24,25-DHL followed by the reduction of the resulting 8,14-diene 3; *ii*) lathosterol, a direct precursor (15) to cholesterol via the 5,7-diene; and *iii*) cholesterol itself. Sterol 4 is an excellent marker for P450$_{14DM}$ activity, and lathosterol has been shown to be an excellent indicator of whole body cholesterol biosynthesis in man.

Under the conditions of our assay, lathosterol is the major C-27 sterol that accumulated in the cholesterol biosynthetic pathway (Table 1). This observation suggests that, under the incubation conditions used in this study, the Δ^7 sterol 5-desaturase is rate-determining in the latter stages of cholesterol biosynthesis in rat liver preparations. The accumulation of significant quantities of lathosterol raises intriguing questions as to the fate of this sterol in vivo. Is the accumulation of lathosterol, a sterol preceding cholesterol in the biosynthetic pathway, simply producing a readily available supply reservoir for cholesterol, or does lathosterol have other, as yet undetermined, functions?

TABLE 3. Inhibition data for sterols 6–16

Inhibitor	Conc.	% 1 + 5[a]	% 4[a]	% [24,25-^{3}H]DHL	% Inhibition[b] 1 + 5	% Inhibition[b] 4	K_i[c]
	μM						μM
6	0	7.6 ± 0.2	40.7 ± 0.9	32.3 ± 0.4			
6	5	1.4 ± 0.1	3.1 ± 0.1	89.6 ± 0.1	81.1	92.3	0.64
6	10	0.7 ± 0.1	1.8 ± 0.4	92.5 ± 0.3	90.3	95.5	
6	20	0.5 ± 0.1	0.8 ± 0.1	94.8 ± 0.3	93.4	98.1	
7	0	6.9 ± 0.1	40.8 ± 0.2	32.9 ± 0.1			
7	5	3.6 ± 0.6	20.8 ± 0.1	63.4 ± 1.0	47.9	48.9	11
7	10	2.1 ± 0.8	15.8 ± 0.3	71.9 ± 0.3	70.3	61.1	
7	20	1.2 ± 0.1	12.2 ± 0.1	78.1 ± 0.2	82.4	70.1	
8	0	6.7 ± 0.1	37.1 ± 0.1	36.1 ± 0.3			
8	5	1.1 ± 0.1	5.3 ± 0.1	86.4 ± 0.7	84.1	85.6	0.61
8	10	0.7 ± 0.1	2.6 ± 0.1	90.7 ± 0.2	89.4	93.0	
8	20	0.4 ± 0.2	1.6 ± 0.1	93.0 ± 0.2	94.6	95.8	
9	0	7.9 ± 0.3	39.1 ± 0.1	32.4 ± 0.3			
9	5	3.0 ± 0.1	13.5 ± 0.2	73.3 ± 0.6	61.7	65.5	2
9	10	1.9 ± 0.1	8.6 ± 0.3	81.6 ± 0.4	72.3	77.9	
9	20	1.2 ± 0.4	5.6 ± 0.3	87.2 ± 0.8	85.4	85.8	
10	0	8.3 ± 0.7	40.2 ± 0.3	32.0 ± 0.2			
10	5	9.4 ± 0.4	28.0 ± 0.1	45.8 ± 0.7		30.4	>55
10	10	9.0 ± 0.2	23.5 ± 0.3	51.9 ± 0.3		41.7	
10	20	5.1 ± 0.11	19.4 ± 0.6	62.5 ± 1.0	38.8	51.8	
11	0	9.2 ± 0.6	29.6 ± 0.1	34.3 ± 0.4			
11	5	9.0 ± 0.2	23.9 ± 0.5	45.7 ± 1.2	2.6	19.3	30
11	10	7.5 ± 0.7	23.7 ± 1.2	49.4 ± 1.0	18.6	20.1	
11	20	5.9 ± 0.1	23.0 ± 1.4	51.5 ± 0.3	35.9	22.4	
12	0	6.5 ± 0.7	28.8 ± 0.7	46.0 ± 0.4			
12	5	2.2 ± 0.1	7.1 ± 0.2	82.5 ± 0.9	65.9	75.3	3
12	10	0.9 ± 0.3	3.1 ± 0.1	89.7 ± 0.1	78.0	89.3	
12	20	0.4 ± 0.1	1.7 ± 0.1	92.8 ± 0.1	93.9	94.2	
13	0	7.2 ± 0.1	40.0 ± 0.7	33.5 ± 0.2			
13	5	6.2 ± 0.9	28.2 ± 0.3	49.7 ± 2.0	14.5	29.5	9
13	10	5.1 ± 0.4	28.4 ± 1.2	50.7 ± 1.9	29.9	28.9	
13	20	4.0 ± 0.6	28.2 ± 0.5	52.3 ± 0.6	44.4	29.3	
14	0	7.3 ± 0.3	38.5 ± 1.2	35.5 ± 1.7			
14	5	5.1 ± 0.5	28.5 ± 0.1	51.8 ± 0.9	30.9	26.1	0.54
14	10	2.3 ± 0.1	24.8 ± 0.3	60.4 ± 0.6	69.3	35.5	
14	20	1.1 ± 0.1	19.7 ± 0.2	68.7 ± 0.3	84.9	48.8	
15	0	8.8 ± 0.1	38.1 ± 0.4	32.5 ± 0.3			
15	5	9.5 ± 1.1	33.4 ± 1.5	37.2 ± 0.4		12.3	>55
15	10	9.4 ± 0.2	34.4 ± 0.1	36.5 ± 0.4		9.6	
15	20	6.4 ± 0.1	41.8 ± 0.1	32.8 ± 0.2	27.3		
16	0	7.0 ± 0.2	40.8 ± 0.2	33.3 ± 0.1			
16	5	8.8 ± 0.4	27.6 ± 0.1	46.9 ± 0.4		32.3	32
16	10	7.9 ± 0.2	26.6 ± 0.7	48.9 ± 0.4		34.9	
16	20	5.7 ± 0.1	26.5 ± 0.3	53.2 ± 0.9	18.9	35.0	
CN^-	0	5.1 ± 0.4	36.4 ± 0.4	40.0 ± 0.8			
CN^-	5	10.4 ± 0.1	29.7 ± 0.9	41.4 ± 1.8		18.3	
CN^-	10	10.4 ± 0.1	29.0 ± 0.2	42.9 ± 0.3		20.2	
CN^-	20	9.9 ± 0.1	29.3 ± 1.1	43.2 ± 0.3		19.3	
AY	0	5.4 ± 0.1	32.3 ± 0.1	36.4 ± 0.2			
AY	5	ND[d]	10.6 ± 0.4	38.6 ± 1.6		63.1	
AY	10	ND	4.8 ± 0.1	37.8 ± 0.6		85.3	
AY	20	ND	3.5 ± 0.9	38.0 ± 2.4		89.3	
KTZ	0	5.8 ± 0.3	40.7 ± 0.9	33.6 ± 0.3			
KTZ	5	1.6 ± 0.2	8.3 ± 0.3	81.4 ± 0.5	72.9	79.5	
KTZ	10	0.7 ± 0.1	3.5 ± 0.5	89.2 ± 0.7	88.3	91.5	
KTZ	20	0.3 ± 0.1	1.1 ± 0.1	91.9 ± 0.4	94.3	97.2	

Assays were performed as described in Assay conditions. All data are averages of duplicate incubations ± SD; CN^-, cyanide; AY, AY9944; KTZ, ketoconazole.

[a] % Sterol is the digitally integrated peak area (dpm) for that sterol, i.e., 4, as a fraction of the total digitally integrated peak areas for all detectable sterols, expressed as a percentage.

[b] % Inhibition is the digitally integrated peak area (dpm) for that sterol, i.e., 4, in the presence of inhibitor, as a fraction of the digitally integrated peak area (dpm) for that sterol in the corresponding control assay, expressed as a percentage.

[c] K_i values were determined as described in reference 4.

[d] ND, not determined; AY9944 does not block 4-demethylation, hence C-27 8,14-diene systems are produced.

Examination of the metabolic profiles for cholesterol biosynthesis in the presence of inhibitor series 6–12 suggested that these compounds were indeed exerting their inhibitory effect, in a dose-dependent manner, on $P450_{14DM}$. This was established by comparing the increase in nonmetabolized [24,25-^{3}H]DHL with the decrease in 14α-demethylated sterol 4. As expected, the decrease in total lathosterol and cholesterol paralleled the decrease in available sterol 4. Hence, it can be concluded that inhibitors 6–12 primarily inhibit $P450_{14DM}$ and do not detectably affect the enzymes downstream of $P450_{14DM}$. Furthermore, these inhibitors have a marked effect on cholesterol biosynthesis, making $P450_{14DM}$ a suitable candidate for therapeutic control of cholesterol biosynthesis in vivo.

The inhibitors in series 13–16 are less potent blockers of both $P450_{14DM}$ and downstream cholesterol biosynthesis. It should be noted that, unlike compounds 6–12, sterols 13–16 lack a nuclear double bond which may reduce their binding affinity for $P450_{14DM}$ and hence their potency as inhibitors. Fischer et al. (16) concluded that since the Δ^8, Δ^7, Δ^6, and saturated dihydrolanosterols were all competitive inhibitors of $P450_{14DM}$ it was unlikely that the nuclear double bond was actually involved in the binding of the substrate to the active site of $P450_{14DM}$. However, the same workers concluded that the position of the nuclear olefinic group affected both the orientation of the 14α-methyl group of lanosterol and reactivity in the 14α-demethylation process. The Δ^8-compound lanosterol was the best substrate and the saturated analogue was not metabolized at all. Aoyama et al. (17) observed that a conformational change occurred in the lanosterol ring system as a result of double bond migration in the B-ring, critically altering the structure of the β-surface. The most striking alterations were in the relative positions of the C-18, C-19, and C-31 methyl groups and the side chain. They concluded that $P450_{14DM}$ may discriminate between different conformations of the β-surface. In our 13–16 series, all compounds contain epoxide systems that alter the sterol ring conformation and, therefore, probably further alter the structure of the β-surface. Hence, it is conceivable that the compounds in series 13–16 are less substrate-like than those of series 6–12, and are correspondingly poorer binders to the active site and therefore poorer inhibitors. Furthermore, it should be noted that compounds 15 and 16 contain neither a 14α-methyl group nor a nuclear olefin, conferring upon these compounds the least substrate-like structure of all the compounds examined. These two compounds were the weakest inhibitors.

Comparison of the efficacy of our inhibitors with ketoconazole, with respect to both $P450_{14DM}$ activity and inhibition of cholesterol biosynthesis, is highly favorable to our steroidal inhibitors (Table 3). Ketoconazole is a nonspecific cytochrome P450 inhibitor, presently in use as an anti-mycotic. It is known to inhibit cytochrome P450 enzymes with central roles in the biosynthesis of the physiologically essential androgens and corticosteroids (18). However, the sterol inhibitors reported here are much more likely to be highly specific inhibitors of $P450_{14DM}$.

Trzaskos et al. (1) have described the use of a radio-HPLC assay to examine the role of $P450_{14DM}$ in lanosterol oxidation. In this report we have described the use of the radio-HPLC assay for examining the effects of steroidal inhibitors on cholesterol biosynthesis from 24,25-DHL. In contrast, Sonoda et al. (19–22) have used the approach of thin-layer chromatography in evaluating the in vitro effects of lanosterol analogues (19), cholesterol analogues (20), oygenated cholesterol derivatives (21), and oxygenated lanosterol derivatives (21, 22) on cholesterol biosynthesis from 24,25-DHL. Thin-layer chromatographic separation of the sterol metabolites resulted in a 4,4-demethyl fraction, from which digitonin-precipitable sterols were isolated and quantitated by liquid scintillation spectrometry. The latter protocol is fundamentally qualitative, as individual sterol metabolites cannot be directly resolved and accurately quantified. Use of the radio-HPLC assay system described in this paper offers the distinct advantages of metabolite resolution, identification, and accurate quantification.

In summary, we have described the effects of a series of steroidal $P450_{14DM}$ inhibitors on C-27 sterol biosynthesis. Furthermore, we demonstrated that several of these sterols were potent blockers of total lathosterol and cholesterol synthesis in vitro, making $P450_{14DM}$ a suitable candidate for therapeutic control of cholesterol biosynthesis in vivo.

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