Substrate-Based Inhibitors of Lanosterol 14α-Methyl Demethylase: I. Assessment of Inhibitor Structure−Activity Relationship and Cholesterol Biosynthesis Inhibition Properties

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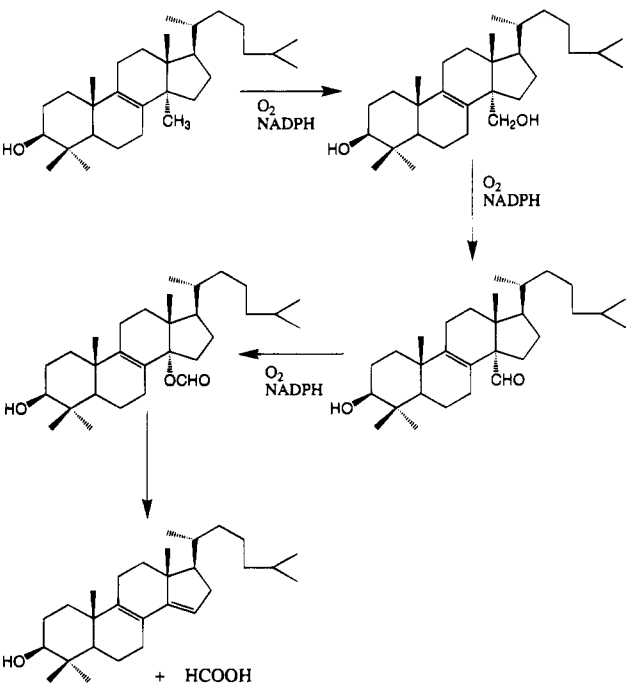
ABSTRACT: A series of 15-, 32-, and 15,32-substituted lanost-8-en-3β-ols is described which function as inhibitors of cholesterol biosynthesis. These agents inhibit lanosterol 14α-methyl demethylase activity as well as suppress HMG-CoA reduction activity in cultured cells. Several of these agents are extremely potent as both demethylase inhibitors and reductase suppressors, while others are more selective in their activities. Selected regio double bond isomers show preference for demethylase inhibition with the following order: $\Delta^8 > \Delta^7 > \Delta^6$ = unsaturated sterols. Comparisons also show that 4,4-dimethyl sterols are always more potent demethylase inhibitors and reductase suppressors than their 4,4-bisnormethyl counterparts. However, evaluation of an extensive oxylanosterol series leads us to conclude that demethylase inhibition and reductase suppression are not parallel in the same molecule. In addition, the oxylanosterols, but not the oxycholesterols, are able to disrupt coordinate regulation of HMG-CoA reductase from the LDL receptor. Thus, oxylanosterol treatment at levels which suppress reductase activity enhances LDL receptor activity. These results demonstrate that compounds can be made which (1) are selective reductase suppressors enabling dissection of the dual inhibitor nature of these compounds and (2) maximize reductase suppression and LDL receptor induction without demethylase inhibition which could lead to novel agents for serum cholesterol lowering.

Lanosterol 14α-methyl demethylase catalyzes the first reaction sequence in the conversion of lanosterol to cholesterol by mammalian microsomes (Gaylor, 1981). During the demethylase catalytic cycle, the enzyme performs three successive stereospecific oxidations on the initial lanosterol substrate resulting in formic acid loss and conjugated diene formation (Scheme 1). These observations have resulted in classification of the lanosterol 14α-methyl demethylase cytochrome P-450 as the lanosterol C14−C32 lyase (Yoshida & Aoyama, 1984; Aoyama et al., 1984, 1987; Trzaskos et al., 1986b).

From a metabolic perspective, the significance of the lanosterol 14α-methyl demethylase as a key enzyme controlling the overall flux of carbon through the cholesterol biosynthetic pathway has become evident in recent years. This prominence is not attributable to direct regulatory features of the demethylase enzyme, but rather to the inherent properties of the oxylanosterol metabolites generated during the course of lanosterol demethylation. Gibbons et al. (1980) first demonstrated that 32-oxygenated lanosterols were potent suppressors of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, when added to cultured cells. Tabacik et al. (1981) extended these observations and showed that the oxygenated lanosterol, 3β-hydroxylanost-8-en-32-al, accumulated in cells with a reciprocal decrease in cholesterol synthesis. Studies from our laboratory have demonstrated that manipulations of the

Scheme 1: Lanosterol 14α-Methyl Demethylase Reaction Sequence



lanosterol 14α-methyl demethylase by enzyme inhibitors or excessive substrate results in significant accumulation of oxygenated lanosterol demethylation intermediates during *in vitro* assays (Trzaskos et al., 1986a, 1987; Favata et al., 1987). The same manipulations of the demethylase in cultured cells fostered oxylanosterol accumulation which

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correlated with a reciprocal suppression of HMG-CoA reductase activity and obligate inhibition of cholesterol biosynthesis (Trzaskos et al., 1987; Saucier et al., 1987). Thus, the collective enzymological and metabolic data support the notion that lanosterol demethylation serves as a point for generating regulatory metabolites which mediate in part overall carbon flux through the cholesterol biosynthetic pathway.

At first glance, the regulatory properties of the oxylanosterol molecules are consistent with those of other oxysterols which make up one component of the multivalent feedback mechanism controlling HMG-CoA reductase activity (Brown & Goldstein, 1980). This process entails a sterol mediator molecule which suppresses reductase activity through a decrease in the amount of reductase protein. Oxygenated lanosterols have been shown to affect HMG-CoA reductase in this manner (Saucier et al., 1987). However, upon closer inspection the data suggest that oxylanosterols regulate reductase through a unique mechanism (Trzaskos et al., 1993; Leonard et al., 1994; Panini et al., 1992; Anderson et al., 1995). It has been shown that decreases in reductase mass do not correlate with decreases in reductase mRNA levels when cells are treated with lanosterol analogs. Thus, reductase expression is regulated by a decrease in translational efficiency of reductase mRNA, resulting in lower rates of reductase synthesis. This contrasts with the reduction in reductase protein brought about by 25-hydroxycholesterol which results from a decrease in transcriptional activity of the reductase gene and an enhancement of the enzyme degradation rate (Brown & Goldstein, 1980).

This finding has important mechanistic implications for cellular cholesterol homeostasis, as it imparts unique regulatory properties to cholesterol biosynthetic intermediates distinct from those of cholesterol-derived origin. By virtue of posttranscriptional control, the ability to disrupt coordinate regulation of HMG-CoA reductase from LDL receptor expression becomes apparent (Panini et al., 1992; Adams et al., 1991; Anderson et al., 1995). On the basis of these findings, the attractiveness of the lanosterol demethylase as a pharmacological target for hypocholesterolemic drug design becomes evident. Thus, we undertook a systematic approach to design mechanism-based inhibitors of the demethylase enzyme.

Our initial strategy was to exploit the first two oxidations in the demethylation cycle, permitting the enzyme to activate potential substrates (prodrugs) to reactive intermediates. It was envisioned that potent, specific demethylase substrate/inhibitors would be good inhibitors of cholesterol biosynthesis for several reasons. First, such compounds would inhibit cholesterol formation at the site of lanosterol demethylation. Second, demethylase inhibitors might cause the accumulation of oxysterol suppressors of HMG-CoA reductase generated *in situ*, leading to HMG-CoA reductase suppression. Third, if the feedback hypothesis were valid, such compounds might in fact be suppressors of HMG-CoA reductase directly, thus mimicking the natural, endogenously synthesized oxysterols, leading to a decrease in cholesterol biosynthesis through suppression of HMG-CoA reductase activity and mevalonate formation.

In this report, we present findings from our inhibitor design studies. The inhibition profiles of various lanosterol analogs toward the lanosterol 14α-methyl demethylase are summarized. In addition, the effects of these compounds upon HMG-CoA reductase activity in cultured cells are presented. Our results show that the demethylase is sensitive to numerous substitutions about the sterol nucleus resulting in potent enzyme inhibition. The majority of these molecules also display potent HMG-CoA reductase suppression activity which contributes to their cholesterol biosynthesis inhibition profile. In the accompanying paper (Trzaskos et al., 1995) the unique properties of selected demethylase inhibitors are described. Results from the evaluation of these agents as hypocholesterolemic drugs will be forthcoming.

MATERIALS AND METHODS

15- and 32-Substituted Sterols. Methods describing the synthesis and physical/chemical characterization of 15-substituted sterols, 32-substituted sterols, and 15,32-disubstituted sterols used throughout these investigations are presented in detail elsewhere (Gaylor et al., 1991).

Sterol Standards. Other sterol standards were prepared as previously described (Shafiee et al., 1986). 24,25-Dihydrolanosterol was prepared from commercial sources by preparative HPLC (Fischer et al., 1989). [24,25-$^3H_2$]-24,25-Dihydrolanosterol was from New England Nuclear (lot 1574-252). All other reagents were of the best grade commercially available and from sources described (Trzaskos et al., 1986a).

Lanosterol 14α-Methyl Demethylase Assay. Kinetic studies were performed with [24,25-$^3H_2$]-24,25-dihydrolanosterol as substrate with various indicated sterols as inhibitors. Sterols were suspended in a total of 5 mg of Triton WR-1339 in reaction tubes prior to the addition of assay buffer (0.1 M potassium phosphate, 0.1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.4) and rat hepatic enzyme source (Trzaskos et al., 1986a,b). Reactions were initiated after a 5-min preincubation at 37 °C by the addition of cofactors (2.0 mM NADPH, 0.3 mM NADH, 10 mM isocitrate, 4 mM $MgCl_2$, and 0.5 unit of isocitrate dehydrogenase). Total assay volume was 0.5 mL. Incubations were terminated by the addition of 0.5 mL of 15% KOH in 95% methanol followed by saponification, extraction, and HPLC analysis of reaction products (Shafiee et al., 1986).

3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (HMGR) Suppression Assay. Compounds were tested for their ability to suppress HMGR activity in a cell-based assay system. Chinese hamster ovary (CHO) cells were divided twice weekly and were maintained in McCoy's 5A medium supplemented with 1% Cab-O-Sil delipidated fetal bovine serum (FBS) (obtained from Gibco Laboratories, Chagrin Falls, OH). Cells were harvested during the logarithmic phase of growth, and cell cultures were prepared by adding 0.5×10^6 cells to each well in a 24-well cluster dish employing 1 mL of the above medium per well. The cell cultures were incubated for 48 h at 37 °C in a 5% CO_2, 95% air environment. The test compounds in a 2.5% suspension of bovine serum albumin (BSA) (fatty acid free) in ethanol were then added to the cultures such that the final ethanol and BSA concentrations in the incubation medium were 0.5% and 0.25%, respectively. Treated cells were incubated with the indicated compounds for 6 h at 37 °C in a 5% CO_2/95% air environment. Control cells and those which received test compound were treated in an identical fashion, except the control cells were incubated with the BSA and ethanol suspension only.

Following treatment, HMGR activity was measured in digitonin-permeabilized cells (Leonard & Chen, 1987).

Specifically, the medium in each well was aspirated, and the cells were rinsed with a 50 mM solution of phosphate-buffered saline (PBS). One milliliter of 30 μg/mL digitonin in CSK buffer [10 mM Pipes (1,4-piperazinediethanesulfonic acid, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, and 1 mM EGTA, pH 6.8] was added to each well and incubated for 10 min at 22 °C to permeabilize the cells. The buffer was carefully aspirated, and the wells were rinsed twice with 1 mL of PBS. HMGR activity was measured directly by adding 75 μL of PIB buffer (50 mM potassium phosphate, 1 mM Na_2EDTA, and 10 mM dithiothreitol, pH 7.4) to each well and incubating the cells for 30 min at 37 °C as described above. The enzyme assay was initiated by the addition of 83 μL of substrate/cofactor mixture such that the final assay contained the following: 0.1 M potassium phosphate, 5 mM dithiothreitol, 20 mM glucose 6-phosphate, 2.5 mM NADP, 0.175 unit of glucose 6-phosphate dehydrogenase, and 105 μM [^{14}C]HMG-coenzyme A (15 DPM/pmol), pH 7.4. The assay mixture was incubated for 30 min at 37 °C, and the reaction was terminated by the addition of 70 μL of [^{3}H]-mevalonic acid (35 000 dpm/assay, 0.15 mg/mL) in 3 N HCl. The reaction mixture was left to lactonize for an additional 30 min at 37 °C or overnight at room temperature.

Reaction products were separated by thin-layer chromatography on silica gel G (Analtech) developed in an unsaturated environment with acetone/benzene (3:2, v:v). The band corresponding to mevalonolactone was identified by exposure to iodine vapor and was scraped into counting vials. The extent of conversion of starting substrate, HMG-CoA, to mevalonic acid was determined by liquid scintillation counting in Biofluor (New England Nuclear). Corrections for recovery and blank values were made for each sample. Protein determinations were made by the Bio-Rad dye binding assay (Bio-Rad) according to the manufacturer's instructions using bovine serum albumin as standard. Cellular protein was solubilized from culture dishes by the addition of 20 μL of 16 N KOH and assayed directly for protein amount. Suppression values are expressed as the amount of compound required to suppress HMGR activity by 50% relative to that of the controls.

Cholesterol Synthesis in Mammalian Cells. Compounds were tested for their ability to decreased *de novo* cholesterol synthesis by measuring the incorporation of radiolabeled acetate into cholesterol in HepG2 cells. To conduct this experiment, HepG2 cells were plated at 0.1×10^6 per well in a 24-well tissue culture plate in 1 mL of Dulbecco's modified Eagle's medium/Hams F12 medium (1:1) supplemented with 10% heat-inactivated FBS, 10 μM Hepes, 1 mM sodium pyruvate, nonessential amino acids (Gibco), and 2 mM glutamine. The cells were incubated for 24 h at 37 °C in a 5% CO_2/95% air environment before washing twice with Hank's balanced salt solution (HBSS) and refeeding with 1 mL of the above medium with 1% Cabosil (Kodak) delipidated serum replacing the FBS. The cells were treated with compounds after 24 h of exposure to the delipidated serum medium. All compounds added to the cells were added so that the final concentration of solvent was 0.45% ethanol and 0.25% BSA per well. The compound in the solvent/BSA was sonicated for 10 s to ensure maximum solubilization before addition to the cells. Control wells received solvent/BSA alone. After incubation for 1 h at 37 °C, 20 μCi/mL [5-^{3}H]-(*RS*)-mevalonolactone (MVAL) (20−40 Ci/mmol) (New England Nuclear) was added per well in ethanol/medium so the final ethanol concentration was 1.2% per well. After 22 h of incubation with the compounds, 2.5 μCi of [1,2-^{14}C]acetate [57 mCi/mmol (NEN)] was added per well for an additional 2 h so that the final concentration of ethanol was 1.6%.

The cells were harvested by washing twice with 5 mL of cold PBS, and 1 mL of stop reagent [15% potassium hydroxide and 100 μg/mL butylated hydroxytoluene (BHT) in 85% aqueous methanol] was added per well. The plate was sonicated in a mild water sonicating bath to release the cells from the bottom of the well. The digested cell extracts were then transferred to 15-mL extraction tubes. Each well was rinsed with 1 mL of PBS, which was added to the appropriate extraction tube. The cell extracts were saponified at 80 °C for 1 h, and the nonsaponifiable lipids were then extracted with petroleum ether. The top solvent fraction was further extracted by passage through a silica SepPak (Waters), which binds all the sterols and free fatty acids. Sterols were eluted with a 5-mL diethyl ether/hexane (1:1) rinse. The eluted sterols were dried under nitrogen, resuspended in ethanol, and counted for radioactivity incorporation. Each sample was analyzed by reverse-phase HPLC at 45 °C using an Ultrasphere Octyl column (Altex Scientific) with a mobile phase of acetonitrile/methanol/water (44.5:44.5:10) at a flow rate of 1.5 mL/min.

The extent of sterol synthesis was determined by the amount of radiolabeled [^{14}C]acetate incorporated into the total sterols. The sterol synthesis profile was determined by analysis of the [^{3}H]sterol profile obtain by HPLC.

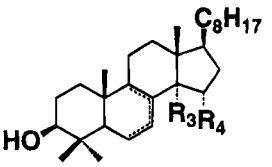
LDL Receptor Activity Determinations. HepG2 cells were grown in 10% fetal bovine serum supplemented medium for 24 h as described above. Cells were washed with HBSS and refed with DMEM and 1% FBS. After 24 h, the test compounds were added in 100% ethanol/2.5% BSA to triplicate wells such that the final concentration of BSA was 0.25%. All compounds were incubated with the cells for an additional 17 h. The cells were then washed with DMEM with 10 mg/mL BSA and incubated with DMEM, 1% delipidated FBS, 120 mM Hepes, and 75 μg of [^{125}I]LDL, with or without 300 μg of cold LDL, for 3 h at 4 °C, after which the cultures were washed 3 times with HBSS and dissolved with 0.1 N NaOH. Aliquots were either counted for [^{125}I]LDL total cell association or measured for protein content by the Lowry method.

RESULTS

Inhibition of 14α-Methyl Demethylase by Various 15- and 32-Substituted 24,25-Dihydrolanosterols and 4,4-Bisnormethyl Analogs. The functionalized lanosterols and 4,4-bisnormethyl analogs were evaluated for lanosterol demethylase inhibition in our standard *in vitro* assay. The sterol concentration required to inhibit control demethylase activity by 50% was determined (IC_{50}). The results are presented in Tables 1−3.

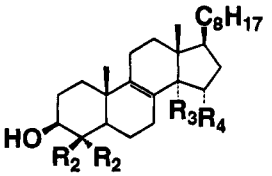
Several general structural features in the substituted lanosterols which influence demethylase inhibitor potency are revealed by the data. First, the Δ^8 isomers are more potent inhibitors than Δ^7 isomers, which are better than Δ^6 and lanostanol analogs (Table 1). Second, 4,4-dimethyl compounds are better inhibitors than the corresponding 4,4-bisnormethyl analogs (Table 2). Third, a free hydroxyl group is preferred over a ketone or ester at C-3 (Table 3). These trends are seen for a variety of analogs which are more artfully differentiated by substitutions at the 15- and 32-positions in the lanosterol molecule (Table 4).

Table 1: Double Bond Isomers vs Activity



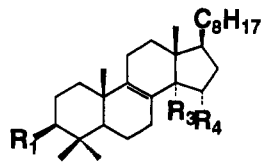
compd. no.	R_3	R_4	Δ	IC_{50} (μM) 14α-DM	HMGR
1	CH=NOH	H	8	1.1	0.04
2	CH=NOH	H	7	3.0	0.13
3	CH=NOH	H	6	>100	0.30
4	CH=NOH	H	sat.	>100	1.10
5	CH_3	=NOH	8	3.6	0.13
6	CH_3	=NOH	7	55.0	0.16
7	CHF_2	H	8	16.0	1.34
8	CHF_2	H	7	37.0	1.42

Table 2: Substitution at C-4 vs Activity



compd. no.	R_2	R_3	R_4	IC_{50} (μM) 14α-DM	HMGR
5	CH_3	CH_3	=NOH	3.60	0.13
9	H	CH_3	=NOH	>100	0.35
1	CH_3	CH=NOH	H	1.10	0.04
10	H	CH=NOH	H	>100	1.30
11	CH_3	CHOHCH=CH_2(*S*)	H	0.75	0.06
12	H	CHOHCH=CH_2(*S*)	H	>100	1.70
13	CH_3	OH	OH	3.00	0.05
14	H	OH	OH	100	0.36

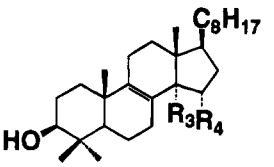
Table 3: Substitution at C-3 vs Activity



compd. no.	R_1	R_3	R_4	IC_{50} (μM) 14α-DM	HMGR
5	OH	CH_3	=NOH	3.6	0.13
15	OAc	CH_3	=NOH	>100	0.10
16	OCOPh	CH_3	=NOH	100	1.00
13	OH	OH	OH	3.0	0.05
17	OAc	OH	OH	1.3	0.60

The results presented in Table 4 highlight the structural diversity which can be incorporated into the lanosterol core structure while still retaining lanosterol demethylase inhibitory activity. Analogs of the natural pendant methyl substrate such as the 32-difluoro analogue **7** are easily accommodated and are potent inhibitors of the demethylase. In contrast, the 15α-fluoro compound **35** does not show any apparent advantage over its hydrogen-containing counterpart. Substituents at position 15 which include an electron-withdrawing group seem to enhance inhibitory potency. This is exemplified by the 15-oxime (**5**) and 15-hydroxyl (**23**)

Table 4: Substitution at C-14,15 vs Activity



compd. no.	R_3	R_4	IC_{50} (μM) 14α-DM	HMGR
18	CH_2SCH_3	=O	<0.50	0.009
19	$CH_2CH=CH_2$	=NOH	2.30	0.020
1	CH=NOH	H	1.10	0.040
20	CH_2OH	OH	0.42	0.045
13	OH	OH	3.00	0.050
11	CHOHCH=CH_2(*S*)	H	0.75	0.060
21	CH_2CH_2OH	H	n.d.	<0.075
22	$CHOHCH_2OH$	H	n.d.	<0.075
23	CH_3	OH	0.50	0.075
24	$CONH_2$	H	n.d.	0.075
25	$CH_2CH_2CH_3$	=O	<1.00	0.130
26	CHOHCH=CH_2(*R*)	H	3.20	0.130
5	CH_3	=NOH	3.6	0.13
27	$CH=CH_2$	H	35.0	0.17
28	CH_3	=O	27.0	0.20
29	$CH_2CH_2CH_3$	=NOH	3.0	0.28
30	CHO	OH	3.0	0.30
31	$CH_2CH=CH_2$	=O	<1.0	0.35
32	SCH_3	=O	n.d.	0.42
33	$NHCO_2Et$	H	>100	0.60
34	CO_2H	H	n.d.	0.81
35	CH_3	F (Δ^7)	>200	1.00
36	NHCHO	H	4.0	1.25
7	CHF_2	H	16.0	1.34
37	CN	H	11.0	1.42
38	CHO	H	>200	22.0
39	CH_2OH	H	7.8	5.0

analogs where a substantial increase in inhibitor potency is observed in compounds containing these modifications.

The most striking increases in inhibitor potency are observed, however, when the 32-substituent is altered from the natural methyl group. For example, the CH=NOH (**1**), $CHOHCHCH_2$ (**11**, **26**), NHCHO (**36**), and CN (**37**) analogs are all more potent inhibitors than the CH_2OH (**39**) counterpart—the most potent natural inhibitor of the demethylase, as well as its most preferred substrate (Fischer et al., 1989). These results indicate that the demethylase is suited to accommodate molecules with rather large, bulky features. The enhanced potency of these analogs suggests that an added degree of binding energy may be coming from heme iron ligation at the enzyme active site. Such an interaction could be monitored spectrally and would be reflected in a type I spectral change for the P_{450} enzyme/substrate complex upon substrate binding (Jefcoate, 1978). Alternatively, heme iron ligation through either nitrogen or oxygen ligation would be reflected in a distinct type II or reverse type I spectrum, respectively. We attempted spectral binding studies on microsomal preparations to help define the nature of enhanced inhibitor potency. Unfortunately, we were unsuccessful in generating spectra, which has been a common problem encountered by other investigators of the demethylase cytochrome (Yoshida & Aoyama, 1984; Yoshida et al., 1987; Tuck et al., 1991). Additional studies employing purified demethylase P_{450} are planned.

HMG-CoA Reductase Suppression by Various 15- and 32-Substituted 24,25-Dihydrolanosterols and 4,4-Bisnormethyl Analogs. The series of functionalized lanosterols were also

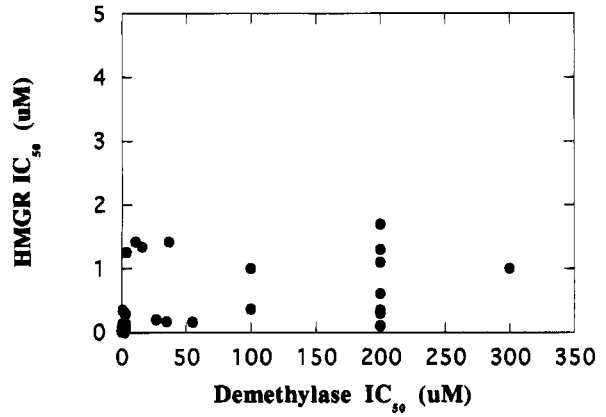


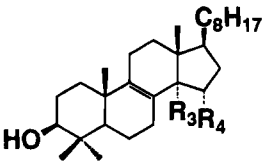
FIGURE 1: Correlation of HMGR suppression and 14α-demethylase inhibition by substituted lanosterols. IC_{50} values for HMGR suppression and 14α-DM inhibition from Tables 1−4 were plotted. The linear correlation for the data is $R^2 = 0.22$, thus indicating no relationship between the two parameters.

evaluated for their ability to suppress HMG-CoA reductase activity in cultured CHO cells. The IC_{50} values for reductase activity suppression are reported in Tables 1−4. The most striking feature revealed by the data is the potency attainable by this class of compounds. For example, the 32-methylmercapto-15-ketone **18** and the 32-vinyl-15-oxime **19** show IC_{50} values of 9.0 and 20 nM, respectively. These values represent a >1000-fold increase in potency over the naturally occurring 3β-hydroxylanost-8-en-32-al **38**, which displays an IC_{50} value of 22 μM.

Also evident in the data is the lack of a direct correlation between HMG-CoA reductase suppression activity and lanosterol 14α-methyl demethylase inhibition. This is more obviously noted in Figure 1. The total lack of correlation ($R^2 = 0.2$) between HMG-CoA reductase suppression and lanosterol demethylase inhibition supports the notion that the two properties are not mutually inclusive. The data also solidify the idea that the two properties may be completely separable, thus permitting HMG-CoA reductase suppression without directly inhibiting lanosterol demethylation. Indeed, compounds displaying these properties have been described (Lin et al., 1995).

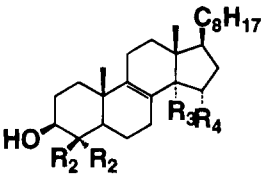
Cholesterol Biosynthesis Inhibition by Selected 15- and 32-Substituted 24,25-Dihydrolanosterols and 4,4-Bisnormethyl Analogs. To assess the ability of selected lanosterol analogs to inhibit cholesterol biosynthesis, we monitored cholesterol synthesis in HepG2 cells. Cells were treated with agents for 24 h, and the extents of cholesterol and total sterol synthesis were determined. The results of these studies are reported in Table 5. The data demonstrate that these agents effectively block sterol biosynthesis from acetate with IC_{50} values in the 0.2−3.5 μM range. These values compare favorably with the oxysterol 25-hydroxycholesterol, but are less impressive than what is seen with lovastatin. The decrease in acetate labeling is sterol specific, as fatty acid labeling indices are unaffected or actually increased in the presence of inhibitors (data not shown). We attribute the decrease in sterol labeling from acetate as a measure of the extent of HMG-CoA reductase suppression. In parallel with the acetate studies, the disposition of mevalonate labeling of sterols was monitored. Mevalonate labeling was used to determine the extent of sterol intermediate accumulation as a result of inhibiting enzymes distal to HMG-CoA reductase. The results reported in Table 5 show that total sterol production from mevalonate is not inhibited as assessed by

Table 5: *In Vitro* Cholesterol Biosynthesis Inhibition Activity of Oxylanosterols



compd. no.	R_3	R_4	IC_{50} (μM) ^{14}C-OAc	^{3}H-MVA
1	CH=NOH	H	2.00	n.d.
19	CH_2OH	OH	3.50	inact.
12	OH	OH	3.50	>10.0
10	$CHOHCH{=}CH_2(S)$	H	0.74	inact.
20	CH_2CH_2OH	H	7.30	inact.
21	$CHOHCH_2OH$	H	0.21	inact.
23	$CONH_2$	H	0.40	n.d.
5	CH_3	=NOH	0.50	n.d.
36	CHO	H	22.0	inact.
	lovastatin		0.03	inact.
	25-hydroxycholesterol		1.00	inact.

Table 6: Induction of LDL Receptor by Oxylanosterols



compd. no.	R_2	R_3	R_4	LDLR induction	dose (μM)
5	CH_3	CH_3	=NOH	+70%	1.0
9	H	CH_3	=NOH	−12%	15.0
11	CH_3	$CHOHCH{=}CH_2(S)$	H	+79%	10.0
12	H	$CHOHCH{=}CH_2(S)$	H	−11%	15.0
40	H	$CHOHCH{=}CH_2(R)$	H	−27%	15.0
	lovastatin			+83%	1.5
	25-hydroxycholesterol			−62%	5.0

radio HPLC analysis. Thus, enzymes in the pathway up to lanosterol formulation are not affected by these agents. This result suggests that the basis for the decrease in cholesterol synthesis observed with the oxylanosterol analogs is primarily due to a decrease in carbon flux through the biosynthetic pathway caused by HMG-CoA reductase suppression and not inhibition of other enzymes. An accumulation of lanosterol to as much as 150% has been observed, however. This reflects the blockade of the lanosterol demethylase caused by these compounds, but it is insufficient to account for the overall decrease seen in cholesterol production.

LDL Receptor Induction by Oxylanosterols. Effects on LDL receptor activity were determined with the 15-oximes **5** and **9** and the 32-vinyl alcohols **11** and **12**. This comparison allowed a distinction to be made between the lanostane nucleus and the cholestane nucleus based upon LDL receptor induction. As shown in Table 6, only the oxylanosterol analogs induce LDL receptor activity. The induction amounts to at least 70%, comparable to that seen with lovastatin. Thus, a distinction between seemingly similar sterol molecules can be made on the basis of the ability of oxylanosterols to disrupt coordinate regulation of LDL receptor activity from HMG-CoA reductase. This property is not observed with oxysterols lacking the 4,4-

gem-dimethyls, indicating that this is a specific effect restricted to oxylanosterols.

DISCUSSION

In this report we have examined a series of 15- and 32-substituted 24,25-dihydrolanosterol analogs and their 4,4-bisnormethyl counterparts as cholesterol biosynthesis inhibitors. These agents were evaluated for four separate, but related, activities: (1) direct inhibition of lanosterol 14α-methyl demethylase, (2) suppression of HMG-CoA reductase activity in cultured cells, (3) cholesterol biosynthesis inhibition resulting from a combination of the first two properties, and (4) LDL receptor induction. Our strategy in inhibitor design was to exploit our knowledge of the lanosterol demethylation cycle. This approach guided our synthetic direction toward more potent, mechanism-based inhibitors of the lanosterol demethylase enzyme. The results presented in this manuscript support the validity of this approach as seen in the correlation between demethylase inhibitor potency and known structural features required for demethylase-dependent lanosterol metabolism. For example, we have shown that our inhibitors demonstrate the same general nuclear double bond specificity as seen with lanosterol substrates; namely, the Δ^8 isomers are preferred (Fischer et al., 1989; Aoyama et al., 1989). Also, a preference for 4,4-dimethyl sterols was seen over the 4,4-desmethyl analogs, which supports the anticipated reaction preference for sterol demethylation in mammalian microsomes (Gaylor, 1981). Bossard et al. (1991) have shown similar results in a series of 32-ethynyllanost-8-en-3β-ols where the Δ^8 olefin was a better inhibitor that the Δ^7, and the 4,4-*gem*-dimethyl sterol was a more potent inhibitor than the 4,4-desmethyl analog. Previous findings have demonstrated that hepatic microsomes can metabolize 14α-(hydroxymethyl)-5α-cholest-7-en-3β-ol and 5α-cholest-8-ene-14α-carbaldehyde to demethylated products (Pascal, 1980; Galli-Kienle et al., 1980). Presumably this demethylation reaction is catalyzed by the lanosterol 14α-methyl demethylase. Although no direct comparisons have been made on substrate specificity, our inhibition data would suggest that the lanosterols would be preferred over the cholestene counterparts.

These studies have also highlighted the preference for the free 3β-hydroxyl for effective demethylase inhibition in the lanosterol series. Esterification reduced inhibitor potency 2-fold compared to the unprotected alcohol. This observation, however, was not repeated when a 14α-normethyl lanosterol was studied. The 4,4-dimethyl-5α-cholest-8-ene-3β,14α,15α-triol **13** displayed increased demethylase inhibitor potency when the 3β-hydroxy function was esterified (**14**). The significance of this observation is difficult to assess, as the triol is not a substrate for the demethylase due to the absence of a 14α-methyl function. One could speculate that the acetate facilitates uptake by membrane preparations used as the demethylase enzyme source, thereby increasing inhibitor strength. Alternatively, the 4,4-dimethyl analog may undergo preferential deesterification by microsomal esterases, liberating a more potent inhibitor.

Perhaps the most intriguing finding from the present study has been recognition of the great diversity in size and nature of the 15- and 32-substitutions which maintain inhibitory properties. These findings extend those of Bossard et al. (1991) and Tuck et al. (1991) who also demonstrated a relative insensitivity of the demethylase active site to size and extent of 32-alkyl group unsaturation. Such findings suggest that the demethylase substrate binding pocket is rather unrestrictive and perhaps rather promiscuous in nature. For example, replacement of the pendant 32-methyl with the oxime **1** or the vinyl alcohol **11** significantly increased inhibitor potency. These molecules displayed IC_{50} values of 1.1 and 0.75 μM, respectively. If one invokes the calculations described by Chou (1974), these values equate to K_i values of 0.83 and 0.56 μM, respectively. Similarly, replacement of the 15α-hydrogen with the 15-oxime **5** or the 15α-alcohol **23** leads to IC_{50} values of 3.6 and 0.5 μM. These are rather potent inhibitors when one compares the affinity of the natural lanosterol substrate, which displays a K_m of 300 μM (Fischer et al., 1989). Inhibitor potency, however, does show some degree of stereospecificity. Comparison of the 32-vinyl alcohols **11** and **26** reveals a preference for the *S*-isomer **11**. A similar preference for the *S*-isomer was noted by Tuck et al. (1991) for the (2′-*S*/*R*)-32-oxiranyl and (2′-*S*/*R*)-32-thiiranyl compounds. Bossard et al. (1991) also showed that the 32-*S* propargylic alcohol was more potent than the 32-*R* derivative. This suggests that the stereochemistry of the interaction within the active site is important for demethylase inhibition. Indeed, we have shown that the degree of selectivity is reflected in the type of inhibition observed with the *R*,*S* isomeric pair [see Trzaskos et al. (1995)].

A second important feature of the 15- and 32-substituted lanosterols has been their ability to suppress HMG-CoA reductase activity. The agents described in this paper show enhanced potency over previously described oxylanosterol analogs (Trzaskos et al., 1993; Leonard et al., 1994; Frye et al., 1994). This is especially true for compounds with bifunctionality such as the 32-methylmercapto-15-ketone **18** and the 32-vinyl-15-oxime **19**, which suppress the reductase at 9 and 50 nM, respectively. These agents represent the most potent suppressors of HMG-CoA reductase reported to date.

The suppressor activity of the oxylanosterols is separable from demethylase inhibition as shown by a lack of correlation between demethylase inhibitor IC_{50} values and those for HMG-CoA reductase suppression. This finding supports the notion that the extent of suppression is not due solely to accumulation of endogenously synthesized oxylanosterol demethylation intermediates resulting from demethylase inhibition, but rather includes direct effects of the compounds upon reductase expression. Indeed, if suppression were due solely to accumulation of demethylation intermediates, one would anticipate a direct correlation between demethylase inhibition and reductase suppression. Additionally, if suppression of HMG-CoA reductase were due to accumulation of endogenous regulators formed at the site of lanosterol demethylase, a biphasic suppression profile should be seen. Previous experience with demethylase inhibitors dictates such effects upon reductase activity (Favata et al., 1987; Trzaskos et al., 1987). Since neither of these two scenarios is supported by our data and it has been shown that the 15-oxime **2** suppresses HMG-CoA reductase in demethylase-deficient AR45 cells (Frye et al., 1994), we conclude that many of the oxylanosterols act as direct suppressors of reductase activity. Adams et al. (1991) have reported similar effects with lanosterol analogs used to study reductase regulation in HepG2 cells. They concluded that mevalonate was required for suppression activity by SKF 104976, thereby implicating a mevalonate-derived product in the regulatory

process. This contrasts with the effects seen with 24(*S*)-25-epoxylanosterol, which suppresses reductase equally well in the presence or absence of mevalonate (Paini et al., 1992). Resolution of this seeming discrepancy will have to await further study.

The combination of lanosterol demethylase inhibition with HMG-CoA reductase suppression is an efficient system to block cholesterol biosynthesis. Indeed, others have described these agents as dual action inhibitors of cholesterol biosynthesis (Frye et al., 1993, 1994). The results from our dual labeling experiments demonstrated that carbon flow through HMG-CoA reductase can be efficiently blocked by these agents. Additionally, we demonstrate that the only sterols other than cholesterol to accumulate in treated cells are lanosterol and dihydrolanosterol. It is our opinion that these sterols would not be detrimental as in the case of earlier simple pharmacological attenuation that resulted in desmosterol accumulation (Blankenhorn & Kuzma, 1961; Laughlin & Carey, 1962), because in the present study further generation of lanosterol becomes self-limiting. With oxylanosterol feedback suppression of mevalonate formation, this class of potentially relevant pharmacological substances would not allow overproduction of any unwanted steroidal intermediate.

Finally, we have demonstrated that the oxylanosterols induce LDL receptor activity in addition to suppressing HMG-CoA reductase. This finding distinguishes the oxylanosterols from the oxycholesterols, as the latter display coordinate regulation of the two activities. The ability to disrupt coordinate regulation is not restricted to the oxylanosterols described here, but also holds true for other lanosterols such as 24(*S*)-25-oxidolanosterol (Panini et al., 1992). We believe that this phenomenon is due to the unique translational control mechanism which oxylanosterols employ to suppress HMG-CoA reductase (Trzaskos et al., 1993; Leonard et al., 1994; Panini et al., 1992; Anderson et al., 1995). By virtue of disrupted coordinate regulation, oxylanosterols should also possess enhanced cholesterol-lowering properties *in vivo* due to LDL receptor induction. Indeed, serum cholesterol lowering has been observed with these molecules upon oral dosing (Trzaskos, 1995). In addition, we have recently described a series of 15-oxalanosterols which maintain HMG-CoA reductase suppression and LDL receptor induction, but are devoid of any activity against the lanosterol 14α-methyl demethylase (Ko et al., 1994; Trzaskos et al., 1994). These agents lower serum cholesterol in animals without accumulation of any intermediate in the cholesterol biosynthetic pathway. Similar steroidal inducers of LDL receptor activity with cholesterol-lowering properties have also been described by Lin et al. (1995). Their utility as pharmaceuticals remains to be established.

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