32-Methyl-32-oxylanosterols: Dual-Action Inhibitors of Cholesterol Biosynthesis

Leah L. Frye,*,† Kevin P. Cusack,† and Deborah A. Leonard‡

Department of Chemistry, Rensselaer Polytechnic Institute, Troy, New York 12180-3590, and Biology Department, West Virginia University, Morgantown, West Virginia 26506-6057

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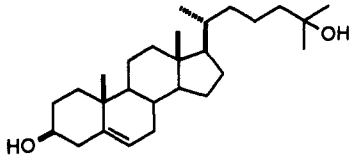
Lanosterol 14α-methyl demethylase (P-450$_{DM}$) is the cytochrome P-450 monooxygenase which oxidatively removes the 14α-methyl group of lanosterol. This demethylation is considered to be a rate-limiting step in the conversion of lanoesterol to cholesterol. The intermediates in this transformation are known to bind very tightly to P-450$_{DM}$ and have been implicated in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity, the rate-limiting enzyme in overall cholesterol biosynthesis. Three 32-methylated analogs of the intermediates generated during the removal of the 14α-methyl group by P-450$_{DM}$, compounds **17a**, **17b**, and **18**, have been prepared and their biochemical activities assessed. All three compounds were found to be direct inhibitors of P-450$_{DM}$. These compounds were also shown to suppress HMGR activity by reducing the level of enzyme protein.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the reductive deacylation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to yield mevalonate.[1] This conversion is the rate-limiting step of overall cholesterol biosynthesis. The activity of HMGR appears to be regulated through a multivalent feedback mechanism mediated by oxysterols along with another metabolite of mevalonate.[2,3] The prototypical oxysterol, 25-hydroxycholesterol (1), appears to regulate HMGR



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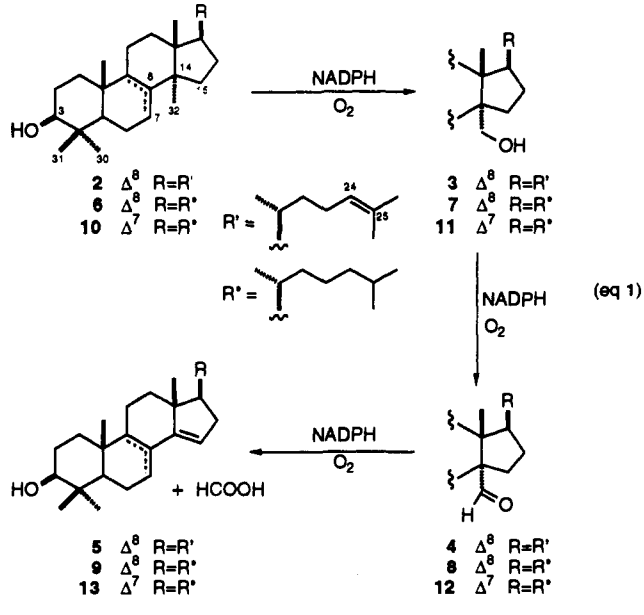
activity via a transcriptional mechanism involving the SRE-1 regulatory sequence.[4] However, recent studies indicate that some oxysterols, particularly oxylanosterols, may be involved in post-transcriptional regulation of HMGR synthesis.[5]

A number of the oxysterols that are suppressors of HMGR activity are catabolites of cholesterol.[6] However, cells which do not oxygenate cholesterol are still capable of regulating the activity of HMGR.[7] HMGR activity and sterol synthesis in these cells can be inhibited by known oxysterol repressors of HMGR.[3,8] These facts strongly suggest that one or more oxysterols which are precursors to cholesterol may be important in the regulation of cholesterol biosynthesis. Oxysterols are generated in the biosynthesis of cholesterol during the oxidative removal of the three "extra" methyl groups (C-30, C-31, C-32) of lanosterol. Lanosterol analogs oxygenated at C-32 (compounds 7 and 8, see eq 1) and their Δ^7-isomers (compounds 11 and 12, see eq 1) have been shown to decrease the activity of HMGR in Chinese hamster lung cells.[8] The 3β,32-diols 7 and 11 have been shown to bind to the oxysterol binding protein and to mediate suppression of HMGR activity.[6] In addition, Trzaskos et al. have recently provided evidence which strongly suggests that aldehyde 8 is an endogenously generated regulator of HMGR activity.[9,10]

32-Oxygenated sterols are formed during the removal of the 14α-methyl group of lanosterol (2) by the cytochrome P-450 monooxygenase, lanosterol 14α-methyl demethylase (P-450$_{DM}$) (eq 1), which catalyzes the first step in the conversion of lanosterol (2) to cholesterol and is considered to be a rate-limiting enzyme in this process.[11] The 14α-methyl group (C-32) of lanosterol (2) is oxidatively removed by P-450$_{DM}$ via three NADPH-O_2 dependent steps.[12] The methyl group is first oxidized to the hydroxymethyl moiety giving compound 3 followed by oxidation to the corresponding aldehyde 4. The nature of the third oxidation, which results in the formation of 8,14-conjugated diene 5 and loss of formic acid, is still unclear but appears to proceed via an enzyme bound peroxyhemiacetal intermediate.[13] Lanosterol (2), 24,25-dihydrolanosterol (6), and lanost-7-en-3β-ol (10) are all substrates for P-450$_{DM}$.[14,15] Lanost-8-en-3β,32-diol (7) and the corresponding Δ^7-



† Rensselaer Polytechnic Institute.
‡ West Virginia University.

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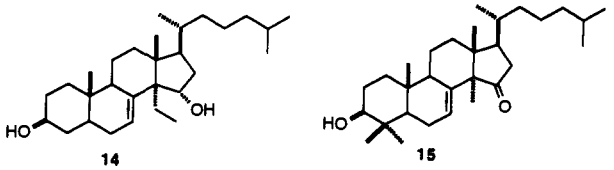
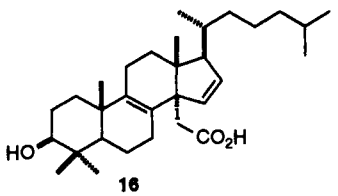
isomer 11 have been shown to bind more tightly to mammalian P-450$_{DM}$ than 24,25-dihydrolanosterol (6) (K_m = 32–35, 5.1, and 5.7 μM for compounds 6, 7, and 11, respectively).[15] In addition, both compounds 7 and 8 have been shown to inhibit the loss of C-32 in rat liver microsomes.[16]

Of the numerous oxysterols which have been studied as suppressors of HMGR activity, a limited number were found to be more potent in the inhibition of overall cholesterol biosynthesis than could be explained by their ability to suppress HMGR activity. Two of these, 14α-ethylcholest-7-ene-3β,15α-diol (14)[17] and 3β-hydroxylanost-7-en-15-one (15),[18] were found to inhibit the conversion of lanosterol (2) (or dihydrolanosterol (6)) to cholesterol which suggests that they are also inhibitors of P-450$_{DM}$. Recently, a novel carboxylic acid analog of lanosterol with Δ^{15}-double bond (compound 16) was also

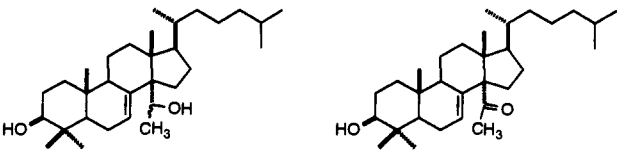




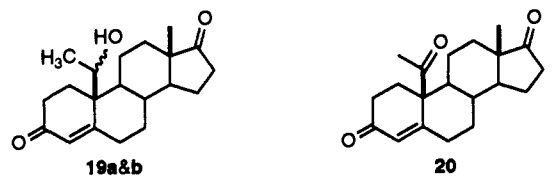
reported to act as both a potent direct inhibitor of P-450$_{DM}$ and an inhibitor HMGR activity.[19] Azole antimycotics are competitive inhibitors of P-450$_{DM}$ which also appear to inhibit HMGR activity by causing the accumulation of the 32-oxylanosterols generated during the removal of the 14α-methyl group of lanosterol by P-450$_{DM}$.[10] The aforementioned compounds are thus inhibitors of cholesterol biosynthesis with two distinct sites of inhibition.

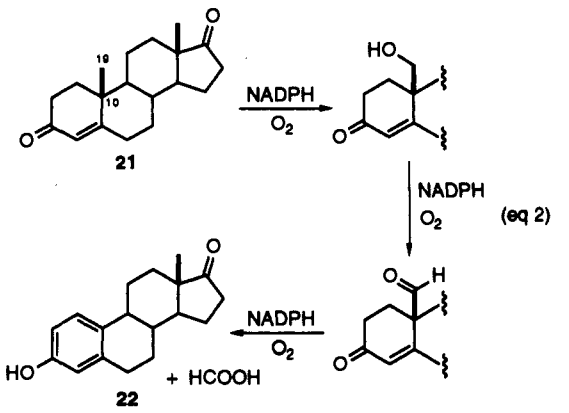
Results and Discussion

Design of Potential Dual-Action Inhibitors of Cholesterol Biosynthesis. Based on the known inhibitory activities of endogenous 32-oxylanosterols,[8,16] we report the preparation of lanosterol analogs **17a**, **17b**, and **18** as potential dual-action inhibitors of cholesterol biosynthesis.[20] These compounds were designed to be competitive inhibitors of P-450$_{DM}$ as well as suppressors of HMGR activity. Compounds **17a**, **17b**, and **18** are methylated analogs of the intermediates generated during the removal of the 14α-methyl group of lanosterol (2) (or dihydrolanosterol (6)) by P-450$_{DM}$, i.e. compounds 3, 4, 7, and 8. Being similar in structure to these natural intermediates, methylated analogs **17a**, **17b**, and **18** may bind tightly to P-450$_{DM}$ and thus act as competitive inhibitors of this enzyme. In addition, they may cause the suppression of HMGR activity either directly or by causing the buildup of the natural intermediates. In the light of the fact that the lanost-7-en-3β-ol (10) is a substrate for P-450$_{DM}$ and that the Δ^{24}-double bond is not necessary for the demethylation,[1,12,15] the 24,25-dihydro-Δ^7-compounds were chosen since they are synthetically more accessible than the 8,24-dienes.



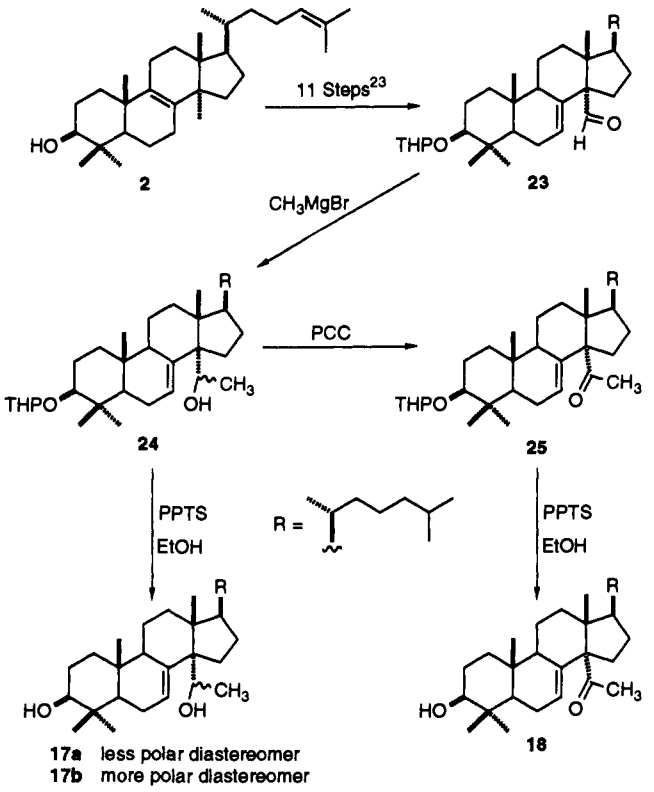
The C-14–C-32 bond of compounds **17a**, **17b**, and **18** should be stable to the lyase activity of P-450$_{DM}$ by analogy to the similar compounds in the aromatase system (i.e. compounds **19a**, **19b**, and **20**).[21] Aromatase is the cytochrome P-450 monooxygenase which oxidatively removes the 10β-methyl group (C-19) of androstenedione (21) to yield estrone (22) (eq 2).[22] The methyl group is removed in a manner analogous to P-450$_{DM}$, i.e. via hydroxylation, oxidation to the corresponding aldehyde, and removal of C-19 as formic acid. Androstenedione analogs **19a**, **19b**, and **20** have been shown by Beusen et al.[21] to be competitive inhibitors of aromatase. In addition, they have demonstrated that the C-10–C-19 bond of these compounds was stable to the lyase activity of aromatase.[21] Considering the similarity of aromatase and P-450$_{DM}$, it is anticipated that the C-14–C-32 bond of lanosterol analogs **17a**, **17b**, and **18** will be stable to cleavage by P-450$_{DM}$. This is important since, if these compounds are to be effective antihypercholesterolemics, they must be resistant to the normally facile metabolism of sterols. The inability to cleave the C-14–C-32 bond should significantly enhance the metabolic stability of compounds **17a**, **17b**, and **18** relative to their naturally occurring analogs. It should be noted that previous approaches to imparting stability to the C-14–C-32 bond of lanosterol analogs have been directed primarily at functionalization at C-15, not at C-32.[17-19]





Chemical Synthesis. Lanosterol analogs **17a**, **17b**, and **18** were prepared as shown in Scheme I from known aldehyde **23**, which is available in 11 steps from lanosterol (2).[23] The methyl group was introduced by treatment of aldehyde **23** with methylmagnesium bromide to yield compound **24**. This THP-protected sterol was then either deprotected to give desired methyl alcohols **17a** and **17b** as a mixture of diastereomers or oxidized with PCC and deprotected to give methyl ketone **18**. The diastereomeric

Scheme I



methyl alcohols **17a** and **17b** were easily separated by preparative normal-phase HPLC. Currently, the absolute stereochemistry at C-32 of the two diastereomeric alcohols is not known. Attempts at obtaining crystals of the alcohols and various ester derivatives which are acceptable for X-ray crystallographic studies have been unsuccessful to date.

Biochemical Evaluation. The inhibition of P-450$_{DM}$ by lanosterol analogs **17a**, **17b**, and **18** was assessed using a modification of the assay developed by Trzaskos and coworkers.[12] Briefly, this assay utilizes dihydrolanosterol (**6**) as the substrate and quantitates the buildup of diene **9** by UV-HPLC. Since rat liver microsomal preparations are used, the pathways by which diene **9** is degraded must be inhibited. The reduction of the Δ^{14}-double bond of diene **9**, the next step in the cholesterol biosynthetic pathway, is inhibited by AY-9944, and the removal of the methyl groups at C-4 is blocked by the addition of cyanide. By varying the concentration of inhibitor at constant substrate concentration (33 μM), this assay consistently gives linear plots of the reciprocal of turnover vs inhibitor concentration. From these plots, the IC_{50} values for compounds **17a**, **17b**, and **18** were calculated to be 0.33, 1.6, and 9.1 μM, respectively (Table I). These results suggest that all three of the methylated lanosterol analogs are direct inhibitors of P-450$_{DM}$ (K_m of 24,25-dihydrolanosterol (**6**) is 33 μM under the assay conditions). Evidence for the inhibition of P-450$_{DM}$ in intact cells by sterols **17a**, **17b**, and **18** was provided by [^{14}C]acetate incorporation studies.[24] Exponentially growing cells were treated with increasing concentrations of inhibitor, pulsed with [1-^{14}C]acetate, and the ^{14}C-radiolabeled nonsaponifiable lipids were analyzed by thin-layer chromatography. Results from representative experiments are shown in Figure 1. All three of the methylated analogs reduced the incorporation of [^{14}C]acetate into material with the chromatographic behavior of C_{27} monohydroxysterols.

Table I. Inhibition of P-450$_{DM}$ and HMGR Activity by Compounds **17a**, **17b**, and **18**

	IC_{50} value (μM)			
	P-450$_{DM}$		HMGR[a]	
compound	in vitro[b]	in vivo[c]	CHO	AR45
17a (less polar diastereomer)	0.33	0.3	3.0	>20
17b (more polar diastereomer)	1.6	0.3	1.5	>20
18	9.1	2.7	1.0	>20

[a] These values were determined from the mean curve of percent inhibition against log concentration, and represent the average of from two to four independent experiments. [b] These values have been published previously[20] and are presented here for comparison. [c] These values indiate the concentration of compound which resulted in the incorporation of equal amounts of [1-^{14}C]acetate into C_{27} and C_{30} sterols during a 2-h incubation and represent the average of two independent experiments.

This decrease was accompanied by an increase in radioactivity in the region of the chromatogram corresponding to the migration of authentic lanosterol (C_{30}) standard. The reduction of incorporation of radiolabel into C_{27} sterols with the concommitant increase in production of C_{30} sterols is consistent with the inhibition of P-450$_{DM}$. Plots of the data from these studies (Figure 1) were used to calculate the concentration of inhibitor which resulted in an equal incorporation of ^{14}C radioactivity into C_{27} and C_{30} sterols. Average values obtained from repetitive experiments are given in Table I. Compounds **17a**, **17b**, and **18** at concentrations up to 20 μM had no effect on the incorporation of [^{14}C]acetate into fatty acids.

An initial screening of the effects of compounds **17a**, **17b**, and **18** on HMGR activity in CHO cells indicates that all three compounds cause a reduction in enzyme activity as compared to control cells. HMGR activity was determined in permeabilized cells or cell sonicates by monitoring the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonate, using [^{3}H]mevalonolactone as an internal standard.[25] The results of representative concentration studies of compounds **17a**, **17b**, and **18** versus HMGR activity and enzyme protein levels are shown in Figure 2. The parallel decline in HMGR activity and protein levels suggests that these compounds suppress HMGR activity by regulating gene expression. The suppression of HMGR protein levels by compounds **17a**, **17b**, and **18** is not due to nonspecific inhibition of cellular protein synthesis. Treatment of cells with these compounds at concentrations up to 5 μM had no effect on the incorporation of [^{3}H]leucine into total acid-precipitable proteins. To test whether compounds **17a**, **17b**, and **18** were acting directly to inhibit HMGR, CHO cell sonicates were preincubated with the compounds prior to assaying HMGR activity. Under these conditions, none of the compounds affected enzyme activity, confirming that these compounds are not direct inhibitors of HMGR. A P-450$_{DM}$-deficient cell line (AR45)[26] was used to test the role of P-450$_{DM}$ in mediating suppression of HMGR by compounds **17a**, **17b**, and **18**. Enzyme activity curves similar to those in Figure 2 were used to calculate the concentration of inhibitor required to reduce HMGR activity to 50% of control values in both CHO and AR45 cells. The values obtained from two to four independent experiments are summarized in Table I. The difference in suppression of HMGR activity in CHO versus AR45 cells for all three compounds suggests that the compounds, by inhibition of P-450$_{DM}$, may cause the accumulation of the natural intermediates **7** and **8** which in turn suppress HMGR activity. This hypothesis is being tested using non-sterol competitive inhibitors of P-450$_{DM}$.[27]

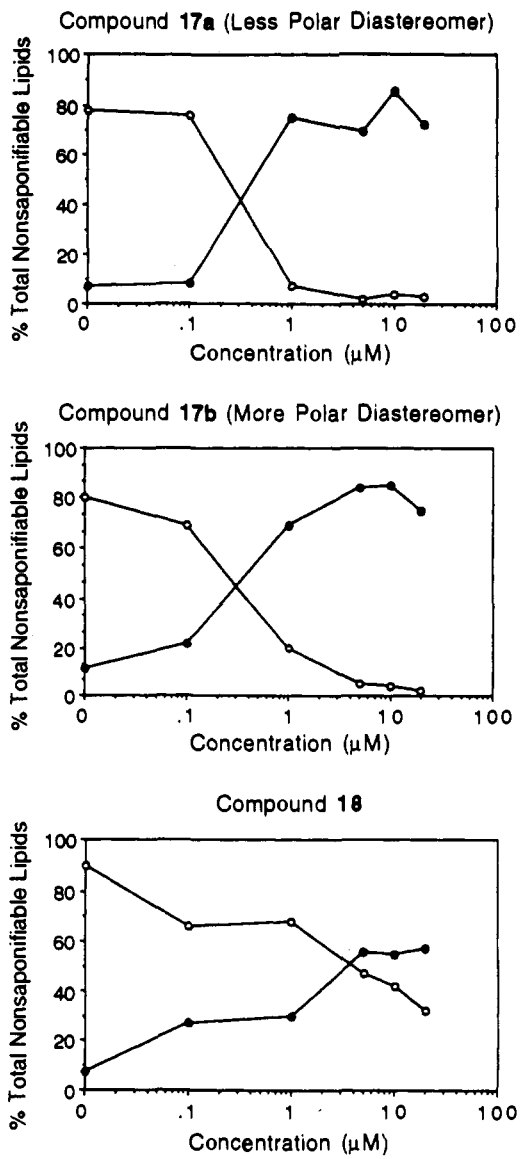


Figure 1. [^{14}C]Acetate incorporation studies in CHO cells. Exponentially growing cells were incubated for 2 h with the indicated concentration of compound and then labeled for an additional 2 h with 0.5 μCi/mL [1-^{14}C]acetate (59 mCi/mmol). Nonsaponifiable lipids were extracted and fractionated by TLC as described in the text. Shown is the percent of total ^{14}C radioactivity comigrating with authentic cholesterol (○) and lanosterol (●) standards. Total incorporation into nonsaponifiable lipids ranged from 40 to 44 dpm/μg protein for control cultures.

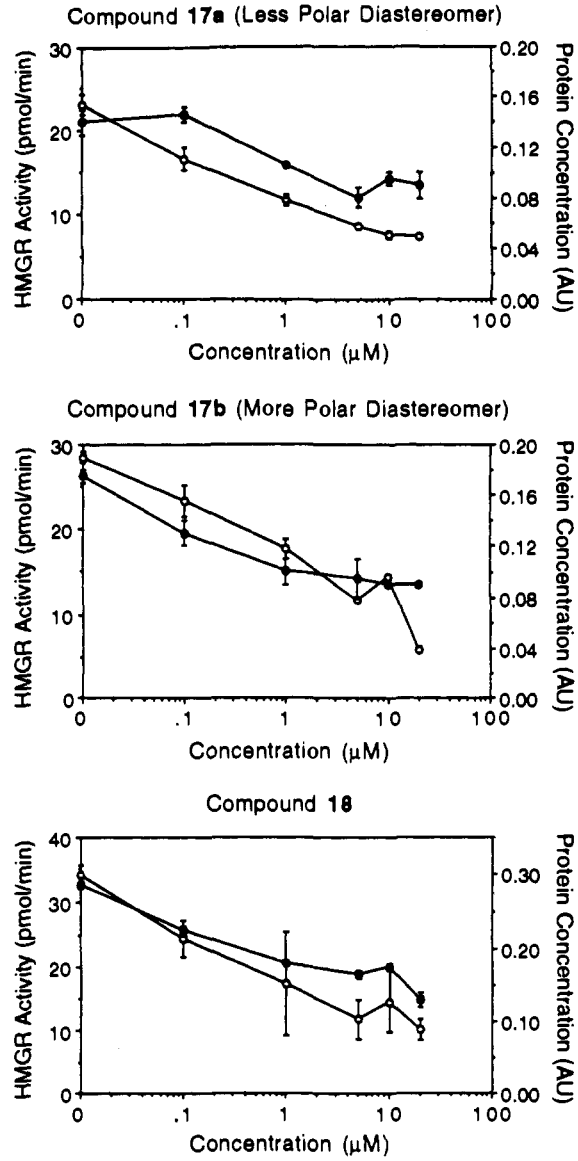


Figure 2. Concentration-dependent suppression of HMGR activity and protein. Cells were treated for 4 h with the indicated concentrations of compound. HMGR activity was determined in permeabilized cells as described in the text. Data are expressed as enzyme activity per 2 × 10^5 cells and represent the mean and standard error for 4 replicates. HMGR protein levels were determined by quantitative immunoblot analysis. Data are expressed in absorbance units and represent the average range of duplicate samples. (○) activity; (●) immunoreactive protein.

Conclusions

32-Methyl-32-oxylanosterols 17a, 17b, and 18 were prepared from known aldehyde 23. All three compounds were found to be direct inhibitors of P-450$_{DM}$. These compounds were also shown to suppress HMGR activity by reducing the level of enzyme protein. Thus, they are acting as dual-action inhibitors of cholesterol biosynthesis. Further experiments to ascertain the absolute configuration of diastereomers 17a and 17b, to determine whether or not compounds 17a, 17b, and 18 are metabolized by P-450$_{DM}$, and to study the mechanism by which these compounds reduce the level of HMGR protein are underway.

Experimental Section

General. ^{1}H NMR spectra were obtained on either a Varian Unity-500 (500 MHz) or a Varian XL-200 (200 MHz) NMR spectrometer with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Perkin-Elmer 298 spectrometer. Direct insertion probe (DIP) chemical ionization mass spectral data were obtained from a Hewlett-Packard HP 5087 GC-MS system. High performance liquid chromatography (HPLC) was performed using a Waters 6000A pump with either a Waters 410 refractive index detector or a Waters Lambda-Max Model 481 variable wavelength UV detector. Preparative HPLC was performed using a Partisil 10 Magnum 9 preparative HPLC column obtained from Whatman. Assays for P-450$_{DM}$ activity in rat liver microsomes utilized a Ultrasphere octyl 5-μm high performance column from Beckman. Liquid scintillation counting was performed using a Beckman LS-5201 liquid scintillation counter and Scintiverse II general purpose cocktail (Fisher). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Spang Microanalytical Laboratories, Eagle Harbor, MI.

Tetrahydrofuran (THF) was distilled from sodium/benzophenone prior to use. The following reagents were purchased from

Aldrich Chemical Co. and were used as received: methylmagnesium bromide, 1,10-phenanthroline, pyridinium chlorochromate, and *p*-toluenesulfonic acid. Lanosterol was obtained from Sigma. 4-Chloro-1-naphthol was obtained from BioRad. The methylmagnesium bromide was titrated using 1,10-phenanthroline as an indicator.[28] Pyridinium *p*-toluenesulfonate was prepared as described by Grieco and coworkers.[29] Silica gel (EM Science Silica Gel 60, 230–400 mesh) was used for all flash chromatography.[30] DL-3-[*Glutaryl*-3-^{14}C]hydroxy-3-methylglutaryl coenzyme A (60 mCi/mmol), L-[3,4,5-^{3}H]leucine (176.5 Ci/mmol), (*RS*)-[5-^{3}H]mevalonolactone (24 Ci/mmol), and sodium [1-^{14}C]acetate were obtained from DuPont-New England Nuclear. The HMGR activity experiments utilized Silica Gel Si-250 TLC plates obtained from Baker. Nitrocellulose membranes (0.45 μm) were obtained from Schleicher and Schuell. Cab-O-Sil M-5 hydrated colloidal silica was from Kodak. McCoy's 5a medium was from Gibco and delipidated fetal bovine serum was from MA Bioproducts. Digitonin (Fisher) was used as a 10 mg/mL stock solution in 50% ethanol. Anti(HMG-CoA reductase) IgG was the generous gift of Dr. Gene C. Ness, University of South Florida, Tampa, FL. The peroxidase conjugated goat antirabbit IgG was obtained from Jackson Immunoresearch. AR45 cells were a generous gift of Dr. Harry W. Chen, DuPont Merck Pharmaceuticals.

Chemical Synthesis. 32-Methyl-3β-(tetrahydropyranyloxy)lanost-7-en-32-ol (24). An oven-dried 5-mL round bottom flask with stirring bar was charged with 3β-(tetrahydropyranyloxy)lanost-7-en-32-al **23**[23] (66.3 mg, 0.13 mmol), equipped with a serum cap and a gas-needle inlet, and flushed with argon. Dry THF (2.0 mL) was introduced and the mixture stirred until all of the steroid was dissolved. The resultant solution was cooled to −78 °C and methylmagnesium bromide (110 mL, 0.32 mmol, 3 M in THF) was added dropwise via syringe. The reaction was allowed to warm to room temperature and stirred for 45 min. The reaction was quenched with aqueous saturated NH_4Cl, the THF removed in vacuo, and the residue extracted well with CH_2Cl_2. The combined organic layers were dried with anhydrous $MgSO_4$. Filtration, concentration, and purification by flash chromatography (SiO_2, 9:1 hexane/ethyl acetate) gave compound **24** (51.4 mg, 75%) as a clear colorless glass: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.40–5.30 (m), 5.25–5.13 (m), 4.79–4.72 (m), 4.62–4.53 (m), 4.38–4.23 (m), 4.04–3.83 (m), 3.57–3.38 (m), 3.30–3.18 (m), 3.12–2.98 (m), 2.15–0.68 (m). (Note: The ^{1}H NMR spectrum is complicated since compound **24** is a mixture of four diastereomers.)

32-Methyllanost-7-ene-3β,32-diols (17a and 17b). A 10-mL round bottom flask with stirring bar was charged with compound **24** (30.8 mg, 58.2 mmol). Absolute ethanol (5 mL) was introduced and the mixture stirred until all of the sterol was dissolved. Pyridinium *p*-toluenesulfonate (PPTS, 22.1 mg, 87 mmol) was added and the mixture stirred overnight. The ethanol was removed in vacuo, and the residue was dissolved in CH_2Cl_2 and washed with distilled water. The organic layer was dried with anhydrous $MgSO_4$. Filtration, concentration, and purification by flash chromatography (6:1 hexane/ethyl acetate) gave compounds **17a** and **17b** (26.0 mg, quant.) as a mixture of diastereomers (ratio of compound **17a** to **17b** was approx. 1.5:1.0). The diastereomers were separated by preparative HPLC (Partisil 10 Magnum 9 preparative column, 3:1 hexane/ethyl acetate, 5 mL/min, RI detector). Compound **17a** (less polar diastereomer): HPLC retention time = 15.01 min; mp 163–164 °C (MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.22–5.16 (m, 1 H, C-7-H), 4.313 (q, *J* = 6.2 Hz, 1 H, C-32-H), 3.224 (dd, *J* = 11.2 Hz, 4.4 Hz, 1 H, C-3-H), 2.1–0.88 (m, 42 H), 0.855 (d, *J* = 6.8 Hz, 3 H, C-27-H), 0.850 (d, *J* = 6.3 Hz, 3 H, C-26-H), 0.695 (s, 3 H, C-18-H)[31] [The resonance for the methyl group attached to C-32 was unequivocally assigned at δ 1.032 (d, *J* = 6.3 Hz) by a decoupling experiment. Irradiation of the quartet at δ 4.313 resulted in collapse of the doublet at δ 1.032 to an apparent singlet.]; IR ($CHCl_3$) 3605 (m), 3500 (w, br), 2955 (s), 2930 (s), 2862 (s), 1600 (w), 1462 (m), 1380 (m), 1368 cm^{-1} (m); MS (CI, isobutane) *m/e* 459 (M + 1, 2%), 441 (M + 1 − H_2O, 100%), 423 (M + 1 − 2H_2O, 68%), 413 (M − C_2H_6O, 38%), 397 (M + 1 − C_2H_5O − H_2O, 92%). Anal. ($C_{31}H_{54}O_2$) C, H. Compound **17b** (more polar diastereomer): HPLC retention time = 20.31 min; mp 121.5–122.0 °C (AcCN); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.38–5.32 (m, 1 H, C-7-H), 3.952 (q, *J* = 6.0 Hz, 1 H, C-32-H), 3.224 (dd, *J* = 11.2 Hz, 4.4 Hz, 1 H, C-3-H), 2.15–0.88 (m, 42 H), 0.865 (d, *J* = 6.4 Hz, 3 H, C-27-H), 0.860 (d, *J* = 6.8 Hz, 3 H, C-26-H), 0.715 (s, 3 H, C-18-H)[31] [The resonance for the methyl group attached to C-32 was unequivocally assigned at δ 1.253 (d, *J* = 6.3 Hz) by a decoupling experiment. Irradiation of the quartet at δ 3.952 resulted in a collapse of the doublet at δ 1.253 to an apparent singlet.]; IR ($CHCl_3$) 3680 (m), 3500 (w, br), 2956 (s), 2932 (s), 2865 (m), 1600 (m), 1462 (m), 1381 (m), 1362 cm^{-1} (m); MS (CI, isobutane) *m/e* 459 (M + 1, 3%), 441 (M + 1 − H_2O, 12%), 423 (M + 1 − 2H_2O, 20%), 415 (M + 1 − C_2H_5O, 17%), 414 (M − C_2H_5O, 12%), 397 (M + 1 − C_2H_5O − H_2O, 100%). Anal. ($C_{31}H_{54}O_2$) C, H. Prior to the biochemical studies, compounds **17a** and **17b**, which had been separated by preparative HPLC as described above, were recrystallized three times and their purity checked by HPLC.

32-Methyl-3β-(tetrahydropyranyloxy)lanost-7-en-32-one (25). An oven-dried 5-mL round bottom flask with stirring bar was charged with compound **24** (15.0 mg, 0.028 mmol). Dry CH_2Cl_2 (2.0 mL) was introduced and PCC (7.0 mg, 0.034 mmol) was added quickly as a solid. The flask was equipped with a serum cap and a gas-needle inlet and flushed with argon. The reaction was stirred at room temperature for 3 h. Celite and anhydrous Na_2SO_4 were added and the mixture stirred for 5 min. The resultant slurry was filtered through Celite, the cake washed well with CH_2Cl_2, and the filtrate concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, 9:1 hexanes/ethyl acetate) to give compound **25** (15.0 mg, 99%) as a white solid: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.66–5.57 (m, 1 H), 4.78–4.69 (m, 0.5 H), 4.60–4.52 (m, 0.5 H), 4.03–3.86 (m, 1 H), 3.56–3.40 (m, 1 H), 3.25 (dd, *J* = 11.6 Hz, 3.6 Hz, 0.5 H), 3.05 (dd, *J* = 11.1 Hz, 3.8 Hz, 0.5 H), 2.55–0.75 (m, 52 H), 0.67 (s, 3 H).

3β-Hydroxy-32-methyllanost-7-en-32-one (18). A 5-mL round bottom flask with stirring bar was charged with compound **25** (15.0 mg, 0.027 mmol). Absolute ethanol (2.5 mL) was added and the mixture stirred until all of the steroid was dissolved. PPTS (20.0 mg, 0.08 mmol) was added as a solid, the flask stoppered, and the mixture stirred at room temperature for 24 h. The ethanol was removed in vacuo. The residue was dissolved in CH_2Cl_2 and washed with aqueous 10% HCl, H_2O, and aqueous saturated NaCl. The organic layer was dried with anhydrous Na_2SO_4 and filtered and the solvent removed in vacuo. The residue was purified by flash chromatography (SiO_2, 5:1 hexane/ethyl acetate) to give compound **18** (12.0 mg, 97%) as a white solid: mp 161.5–162.0 °C (Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.65–5.58 (m, 1 H, C-7-H), 3.224 (dd, *J* = 11.2 Hz, 4.4 Hz, 1 H, C-3-H), 2.50–2.40 (m, 1 H), 2.23–2.12 (m, 1 H), 2.12–1.93 (m, 5 H, includes singlet for C-32-CH_3 at δ 2.022), 1.90–0.88 (m, 34 H), 0.858 (d, *J* = 6.4 Hz, 3 H, C-27-H), 0.852 (d, *J* = 6.3 Hz, 3 H, C-26-H), 0.673 (s, 3 H, C-18-H);[31] IR ($CHCl_3$) 3610 (m), 2960 (s), 1685 cm^{-1} (m); MS (CI, isobutane) *m/e* 457 (M + 1, 100%), 439 (M + 1 − H_2O, 59%), 414 (M + 1 − C_2H_3O, 20%). Anal. ($C_{31}H_{52}O_2$) C, H. Prior to the biochemical studies, compound **18**, which had been purified by flash chromatography as described above, was recrystallized three times and its purity checked by HPLC.

Biochemical Evaluation. Assay of Lanosterol 14α-Methyl Demethylase (P-450$_{DM}$) Activity. The assay of P-450$_{DM}$ developed by Trzaskos et al. was used in these studies.[12] Briefly, dihydrolanosterol (**6**) (33 μM) and varying concentrations of the potential inhibitors (suspended with the aid of Tyloxapal) were incubated with rat liver microsomal protein, 0.2 mM NAD, 0.1 mM NADH, 0.3 mM NADP, 2.0 mM NADPH, 10 mM isocitrate, isocitrate dehydrogenase (0.25 units/mL final volume), 0.4 mM magnesium chloride, 50 μM AY-9944, and 1 mM sodium cyanide in 0.1 mM phosphate buffer (containing 1 mM glutathione, 0.1 mM EDTA, pH 7.4, 20% glycerol) at 37 °C for 45 min. The reactions were stopped by the addition of 15% potassium hydroxide (w/v) in 95% methanol. The resultant mixtures were heated in boiling water for 30 min and the nonsaponifiable material was extracted with petroleum ether. The petroleum ether was blown off with nitrogen, the residue was dissolved in absolute ethanol, and the lanosta-8,14-dien-3β-ol (**9**) content was determined by UV-HPLC (45:45:10 acetonitrile/methanol/water, 1.5 mL/min, Ultrasphere octyl column).

Cell Culture. Chinese hamster ovary (CHO-K1) cells were grown in a 5% CO_2 atmosphere in modified McCoy's 5a medium

supplemented with 1% delipidated fetal bovine serum. Cultures were routinely passaged twice weekly by trypsinization. Test compounds were added as BSA suspensions from stock solutions in absolute ethanol. The final concentration of BSA was 0.5 mg/mL and the final ethanol concentration did not exceed 0.5%.

[^{14}C]Acetate Incorporation into Saponifiable and Nonsaponifiable Lipids. The [^{14}C]acetate incorporation studies were carried out as described previously.[24] Briefly, exponentially growing cultures were labeled with [1-^{14}C]acetate and harvested into ice-cold saline. An equal volume of 15% KOH in 90% methanol containing 100 μg/mL BHT was added and samples were saponified at 85 °C for 30 min. Nonsaponifiable lipids were extracted with at least 30 volumes of petroleum ether, washed once with 3% Na_2CO_3 and twice with water, and dried under nitrogen. Extracts were analyzed by silica thin-layer chromatography in ethyl acetate/*n*-hexane (15:85). To measure incorporation into fatty acids, the aqueous phase was acidified and extracted with petroleum ether, and an aliquot counted in a liquid scintillation counter.

HMG-CoA Reductase Enzyme Activity. This assay has been described in detail previously.[25] Briefly, cells were plated in 24-well cluster plates, grown for 2 days, and permeabilized by incubation for 5 min at room temperature with 30 μg/mL digitonin in cytoskeletal (CSK) buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES, pH 6.8). The digitonin solution was removed, and the permeabilized cells were incubated for 20 min at 37 °C in preincubation buffer (50 mM phosphate buffer, pH 7.4, 10 mM dithiothreitol, 1 mM EDTA). Alternatively, frozen cell pellets were sonicated in preincubation buffer and incubated a 37 °C for 20 min. Reductase activity was determined by monitoring the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonolactone, using [^{3}H]mevalonolactone as an internal standard. The substrate and product were separated by thin-layer chromatography on Silica Gel Si-250 plates, visualized with I_2 vapors, scraped, and counted. Protein concentrations were determined with Bio-Rad protein reagent.

Immunoblot Analysis of HMGR Protein. Quantitative immunoblot analysis of cells was performed as described previously.[25] Cells were solubilized by scraping directly into SDS-urea sample buffer, heated at 90 °C for 3 min, and frozen at −80 °C. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% slab gels. Proteins were electroblotted onto a 0.45-μm nitrocellulose membrane in 49.6 mM Tris, 384 mM glycine, 20% methanol, 0.0375% SDS at 0.4 A for 16 h. Membranes were blocked by washing in 5% nonfat dry milk in TBS/Tween (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5). HMGR was detected using a polyclonal anti(HMGR) antibody, and visualized using a peroxidase-conjugated second antibody. Bands were visualized with 4-chloro-1-naphthol according to the manufacturer's instructions. Immunoblots were quantitated by scanning with a LKB laser densitometer and immunoreactive protein is expressed as the area under the absorbance curve.

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