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Modulation of 3-Hydroxy-3-methylglutaryl-CoA Reductase by 15α-Fluorolanost-7-en-3β-ol

A MECHANISM-BASED INHIBITOR OF CHOLESTEROL BIOSYNTHESIS*

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The chemical synthesis and metabolic characteristics of the lanosterol analogue, 15α-fluorolanost-7-en-3β-ol, are described. The 15α-fluorosterol is shown to be a competitive inhibitor of the lanosterol 14α-methyl demethylase (K_i = 315 μM), as well as substrate for the demethylase enzyme. Metabolic studies show that the 15α-fluorosterol is converted to the corresponding 15α-fluoro-3β-hydroxylanost-7-en-32-aldehyde by hepatic microsomal lanosterol 14α-methyl demethylase but that further metabolic conversion to cholesterol biosynthetic intermediates is blocked by virtue of the 15α-fluoro substitution. When cultured cells are treated with the fluorinated lanosterol analogue, a decrease in 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity and immunoreactive protein was observed. However, when the lanosterol 14α-methyl demethylase-deficient mutant cell line, AR45, is treated with the fluorosterol, no effect upon HMG-CoA reductase is observed. Thus, metabolic conversion of the sterol to its 32-carboxaldehyde analogue by the lanosterol 14α-methyl demethylase is required for HMG-CoA reductase suppressor activity. Measurement of HMG-CoA reductase mRNA levels in 15α-fluorosterol-treated Chinese hamster ovary (CHO) cells reveals that mRNA levels are not decreased by the sterol as would be expected for a sterol regulator of HMG-CoA reductase activity. The decrease in HMG-CoA reductase protein is due to inhibition of enzyme synthesis, suggesting that the 15α-fluorosterol reduces the translational efficiency of the reductase mRNA. Measurements of the half-life of HMG-CoA reductase show that, in contrast to other oxysterols, the 15α-fluorolanostenol does not increase the rate of degradation of the enzyme. Collectively, these data support the premise that oxylanosterols regulate HMG-CoA reductase expression through a post-transcriptional process which may be distinct from other previously described sterol regulatory mechanisms.

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)[1] reductase (EC 1.1.1.34) is the rate-limiting enzyme governing cholesterol biosynthesis and the synthesis of other isoprenoids in mammalian cells (1). It has been suggested that regulation of this important enzyme in cellular metabolism is under the control of a multivalent feedback process which involves both end product sterol, as well as other non-steroidal mevalonate-derived products (2). Results to date demonstrate that sterol-dependent regulation of reductase activity is due primarily to a transcriptional control process which affects the level of reductase mRNA (3, 4), although less dramatic post-transcriptional control by sterols has been observed (5). Conversely, the non-steroidal regulation of reductase has been shown to involve post-transcriptional mechanisms which entail enhanced enzyme degradation and decreased translational efficiency of reductase message (6, 7). Thus, the differences between the two control processes are highlighted in terms of mechanism as well as affector molecule.

Recently there has been renewed interest as to whether cholesterol or some oxysterol precursor or product of cholesterol metabolism is responsible for the observed steroidal regulation of HMG-CoA reductase activity. Generation of regulatory oxysterols by means of a side pathway through epoxycholesterol has been proposed as an alternative to cholesterol as the natural regulator of reductase activity (8–11). Similarly, oxysterol intermediates generated along the normal pathway of cholesterol biosynthesis have been shown to modulate the reductase in a manner consistent with their involvement in the regulatory process (12, 13). In the latter case, it has been demonstrated that 3β-hydroxylanost-8-en-32-al which arises during the course of lanosterol 14α-methyl demethylation can serve to regulate HMG-CoA reductase activity when carbon flux through the sterol pathway is abnormally high (13). Mechanistic studies have also demonstrated that accumulation of 3β-hydroxylanost-8-en-32-al is favored over demethylated end product under conditions when lanosterol substrate concentrations are saturating (14–16), thus supporting the cellular observations on metabolic formation with kinetic data. Similarly, it has been suggested that the level of this same or comparable intermediate may decrease under conditions when substrate pools favor carbon flow to cholesterol (17). Collectively, these observations support the notion that accumulation of 3β-hydroxylanost-8-en-32-al *in situ* may be the result of a designed integrated regulatory process which serves to control endogenous cholesterol biosynthesis under normal physiological fluxes of carbon flow-through the sterol pathway.

The importance of the 3β-hydroxylanost-8-en-32-al in the regulation of HMG-CoA reductase and a detailed understand-

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[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; TMS, trimethylsilyl; CHO, Chinese hamster ovary; NOE, nuclear Overhauser effect; DLSP, delipidated fetal bovine serum.

ing of its mechanism have been hampered, however, by inadequate experimental systems which allow one to monitor specific effects of the endogenously generated sterol metabolite. One approach which we have taken to solve this dilemma has been through the generation of cholesterol biosynthetic mutants which allow dissection of the process genetically (18). A second and equally important approach is to use synthetic substrates. The latter approach has suffered in the past, however, from the inherent problem that synthetic metabolites are ultimately converted to cholesterol as a consequence of normal metabolism. To circumvent this problem, we have designed and synthesized a lanosterol analogue which can only undergo limited metabolism by the lanosterol 14α-methyl demethylase of the cholesterol biosynthetic pathway. The compound described, 15α-fluorolanost-7-en-3β-ol, by virtue of its 15α-fluoro substitution, is incapable of undergoing a complete demethylation cycle and subsequent conversion to cholesterol (19, 20) (Scheme I). This inability to be converted to cholesterol makes the 15α-fluorolanosterol an ideal agent to study the regulatory properties and mechanism of oxylanosterol formation and control of HMG-CoA reductase activity in cultured cells.

In this report, we describe the metabolic characteristics of the 15α-fluorolanostenol and conditions where the fluorinated sterol is metabolized to its aldehyde analogue. Additionally, we demonstrate that the 15α-fluorosterol is an active suppressor of HMG-CoA reductase activity in wild-type, but not lanosterol 14α-methyl demethylase deficient, CHO cells. Most interestingly, the regulation of HMG-CoA reductase by the fluorinated lanostenol in cell culture experiments is shown to be due to a post-transcriptional process analogous to non-steroidal reductase regulators and not transcriptional control as would be expected for a sterol. These results demonstrate that cholesterol precursor sterols regulate HMG-CoA reductase and cholesterol biosynthesis by a mechanism distinct from that of cholesterol and oxycholesterol metabolites. These findings bring into question the identity of the putative HMG-CoA reductase non-steroidal regulator which functions as a post-transcriptional modulator of reductase expression.

EXPERIMENTAL PROCEDURES

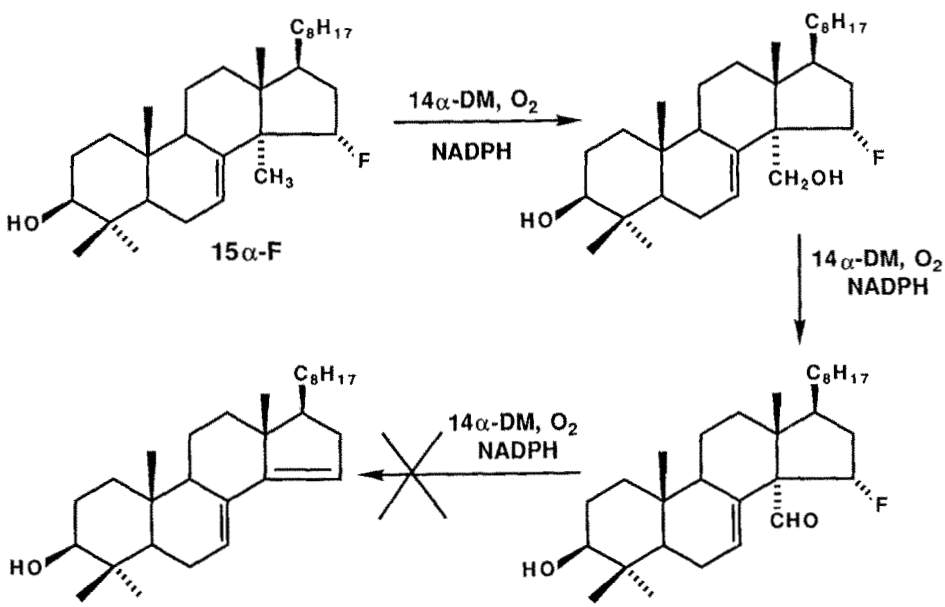
Methods

Lanosterol 14α-Methyl Demethylase Assay—Lanosterol 14α-methyl demethylase was assayed as described previously (16). Kinetic analyses required the following assay modification. Toluene stock solutions containing 1 μCi of [24,25-^{3}H]24,25-dihydrolanost-8-en-3β-ol substrate, 41.7–250 nmol, in addition to 0, 50, or 100 nmol of 15α-fluorolanost-7-en-3β-ol for each substrate concentration, were evaporated in the bottom of each assay tube under N_2 to remove all toluene. To each tube 5.0 mg of Triton WR1339 was added in acetone to dissolve the sterols and then once again dried under N_2 to form a detergent-sterol film in the bottom of the tube. Dimethyl sulfoxide, 50 μl, was added to each tube and vortex-mixed to suspend the detergent-sterol matrix followed by additions of 25 μl of 1.0 mM AY9944 (in a 10% propylene glycol) and 10 μl of 25 μM sodium cyanide. Two mg of rat liver microsomes prepared from male Sprague-Dawley rats that were fed a 3% cholestyramine diet (16) were added to each tube in 390 μl of a 100 mM KxPO4, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol buffer. The microsomes and contents listed above were equilibrated at 37 °C for 5 min and the assay initiated with 25 μl of a 20 mM NADPH/isocitrate dehydrogenase generator mix (16). All other aspects of stopping the assay, saponification, extractions, and analysis by reverse-phase HPLC were as described (16).

Metabolism of 125 nmol of lanost-7-en-3β-ol or 15α-fluorolanost-7-en-3β-ol were performed under the same conditions as described above with the exception that 24,25-dihydrolanosterol was omitted. The extracted nonsaponifiable sterols were split in equal portions and derivatized by the two schemes listed below and analyzed by GC/MS.

Derivatization of Sterols for GC/MS Analysis—The C32-oxysterol products of lanost-7-ene-3β-ol and 15α-fluorolanost-7-ene-3β-ol are unstable during GC/MS analysis, since they decompose to their respective Δ8(14) unsaturated sterol under the high temperature conditions employed during analysis (21). Therefore, separate procedures are described for characterizing the 3β,32-diol sterols *versus* the 3β-hydroxy-32-aldehyde sterols by GC/MS. The first procedure requires that 3β-hydroxy-32-aldehyde sterols be reduced to a 32-hydroxy group prior to derivation. The sterol sample was dissolved in 500 μl of methanol at room temperature, and 5.0 mg of $NaBH_4$ was added and allowed to react for 3 h. Water, 2 ml, was then added to quench the reaction, and the sterols were extracted with 10 ml of petroleum ether. The petroleum ether extract was evaporated under N_2 gas and the reduced sterols subsequently derivatized to their respective trimethylsilyl ethers. The reduced sterols were dissolved in 200 μl of dry pyridine and 200 μl of "Tri-Sil TBT" reagent (Pierce Chemical Co.), capped under N_2 gas, and reacted overnight at 80 °C. The TMS reaction mix was then dissolved in 10 ml of petroleum ether and backwashed with 2 ml of 10% solution of sodium bicarbonate. The organic phase was removed and evaporated to dryness under N_2. The final sterol product was dissolved in 250 μl of toluene, and 4.0-μl aliquots were analyzed by GC/MS as described below.

The alternate method for derivatizing 3β,32-diol sterols as well as their respective substrates and demethylated products was accom-



SCHEME I. **15α-Fluorolanost-7-en-3β-ol metabolism as catalyzed by the lanosterol 14α-methyl demethylase.** Metabolism of the 15α-fluorolanosterol proceeds through aldehyde formation. Further metabolism of the lanostenol requires 15α-proton loss which is prevented by the 15α-fluoro substitution.

plished by performing the "Tri-Sil TBT" reaction as described here without $NaBH_4$ reduction. As above, the final derivative was dissolved in 250 μl of toluene and 4.0 μl was analyzed by GC/MS.

GC/MS Analysis of Sterols—Derivatized sterols were analyzed by GC/MS employing a Hewlett-Packard 5890 gas chromatograph equipped with 5970 mass selective detector (14). A capillary column of DB17-30W (J & W Scientific, Inc.) was used for sterol separations with helium carrier gas and the following temperature program: starting temperature, 235 °C; ramp rate, 5 °C/min; final temperature, 265 °C. Total ion chromatograms were run in the ion range of 50–800 atomic mass units.

Cell Culture—CHO cells were cultured in McCoy's 5A medium (modified) supplemented with 1% Cab-O-Sil (Kodak) delipidated fetal bovine serum (DLSP) as described previously (13). Cells were seeded at a density of 0.3×10^6 in 60 × 15-mm tissue culture dishes (Costar) in 4 ml of the above medium 48 h prior to the start of each experiment (13).

AR45 cells, a CHO cell mutant deficient in lanosterol demethylase, were cultured in McCoy's 5A medium (modified) supplemented with 2.5% fetal bovine serum as described (18). Cells were washed twice with Hanks' balanced salt solution (GIBCO) before replacing the medium with 4 ml of McCoy's 5A medium (modified), 1% DLSP, 18 h prior to the start of experiment.

Determination of HMG-CoA Reductase Activity, Immunoreactive Protein, and Quantitation of mRNA Levels in Sterol-treated CHO Cells—HMG-CoA reductase activity and immunoreactive protein measurements were performed on cell homogenates exactly as described previously in detail (13) or on permeabilized cell cultures as reported by Leonard and Chen (22). Immunoblots were quantitated by scanning with an LKB UltroScan L densitometer.

HMG-CoA reductase mRNA levels were quantified by Northern blot analyses of poly(A^+) RNA. For these determinations, cells were seeded in 150-mm tissue culture dishes (Costar) at 0.9×10^6/dish in 20 ml of McCoy's 5A (modified) medium containing 1% DLSP. Cells were allowed to grow for up to 5 days at 37 °C in 5% $C0_2$. On the day of treatment, the medium was replaced with fresh medium, and cells were treated with sterols suspended in 2.5% bovine serum albumin in 5% ethanol such that the final ethanol concentration did not exceed 0.5% (v/v). All treatments were done in triplicate. The treatment was continued for 16 h at which time cells were harvested by first removing culture medium and washing with 3 × 10 ml of cold (4 °C) 50 μM Tris-HCl, 155 μM NaCl, pH 7.4 (Tris/NaCl buffer). A small portion (14 cm^2) of each culture which was removed by scraping into 0.7 ml of Tris/NaCl buffer for activity measurements and immunoblots. Total RNA was isolated from the remainder of the cultures by guanidine isothiocyanate extraction and CsCl gradient centrifugation, with further fractionation by oligo(dT)-cellulose chromatography (23). Poly(A^+) RNA (2.5 μg) quantitated spectrophotometrically was electrophoresed in 0.66 M formaldehyde, 1% agarose gels (23). Following transfer to nitrocellulose filters, hybridizations were performed with the following nick-translated [α-^{32}P]dATP-labeled cDNA probes (23): the *Bam*HI insert of the HMG-CoA reductase plasmid pRed227 (obtained from ATCC); the *Pst*I insert of the β-actin cDNA (24); and the *Pst*I insert of the gluteraldehyde phosphate dehydrogenase plasmid. pGAPDH plasmid (a kind gift from K. Hastings and C. P. Emerson, Jr. to P. Benfield, Du Pont) (25). The latter two probes were used as internal standards to quantitate mRNA recovery. Autoradiography at −70° for 3–24 h was used to detect the location of radioactivity on filters, and mRNA quantitation was done by cutting individual bands from the filter and counting in a liquid scintillation counter. Similar results were obtained by densitometric scanning of autoradiograms.

HMG-CoA Reductase Synthesis and Degradation—Exponentially growing cells were labeled in medium containing [^{35}S]methionine (40 μCi/ml, 40 Ci/mmol). For synthesis measurements, cells were pulsed-labeled with [^{35}S]methionine for 30 min at various times after treatment with sterols. For degradation measurements, untreated cells were labeled for 2 h with [^{35}S]methionine and then cell monolayers were rinsed twice and incubated in medium containing 10 mM unlabeled L-methionine with or without oxylanosterol. Cells were harvested in Tris/NaCl and frozen at −80 °C. HMG-CoA reductase was immunoprecipitated essentially as described by Tanaka *et al.* (5). HMG-CoA reductase was immunoprecipitated with polyclonal anti-reductase IgG. Immunoprecipitates were solubilized at 37 °C in SDS-urea sample buffer and analyzed by electrophoresis on SDS, 10% polyacrylamide slab gels [^{35}S]methionine-labeled proteins were visualized by fluorography using EN^{3}HANCE (Du Pont-New England Nuclear). Radiolabeled HMG-CoA reductase was quantitated by cutting the appropriate band from the gel, solubilizing the slice with Protosol (Du Pont-New England Nuclear), and counting in a liquid scintillation spectrophotometer. Background radioactivity was determined with preimmune rabbit serum. The half-life of HMG-CoA reductase was calculated from the slope of a semi-log plot of disintegrations/min in reductase *versus* time of chase.

Protein Determinations—Protein was determined by the Bio-Rad dye binding assay according to the manufacturer's directions employing bovine serum albumin as a standard.

Materials

Preparation of 3β-Benzoyloxy-15α-fluorolanost-7-ene (**2**)—The starting material (see Scheme II) used to prepare the desired 15α-fluorolanost-7-en-3β-ol was the previously reported 3β-benzoyloxy-lanost-7-en-15α-ol (26–29). Diethylamino sulfur trifluoride (5.75 ml, 46 mmol) was added to a solution of 3β-benzoyloxylanost-7-en-15α-ol (1, 1.6 g, 2.92 mmol) in dry (distilled from phosphorus pentoxide) dichloromethane (100 ml) (30) under argon at −78°. The reaction was stirred at −78 °C for 0.5 h, then diluted with dichloromethane (100 ml). Aqueous sodium bicarbonate was added until the aqueous phase was neutral, and the mixture was extracted with toluene:ethyl acetate, 1:1 (3 × 100 ml). The combined organic solutions were dried over anhydrous magnesium sulfate, filtered, and solvents removed in vacuo to give 1.7 g of a complex mixture.

Purification by chromatography on silica gel (eluant 5% ethyl acetate in hexane), followed by HPLC on silica gel (eluant 25% toluene in hexane), yielded 294 mg (18% yield) of 3β-benzoyloxy-15α-fluorolanost-7-ene (**2**).

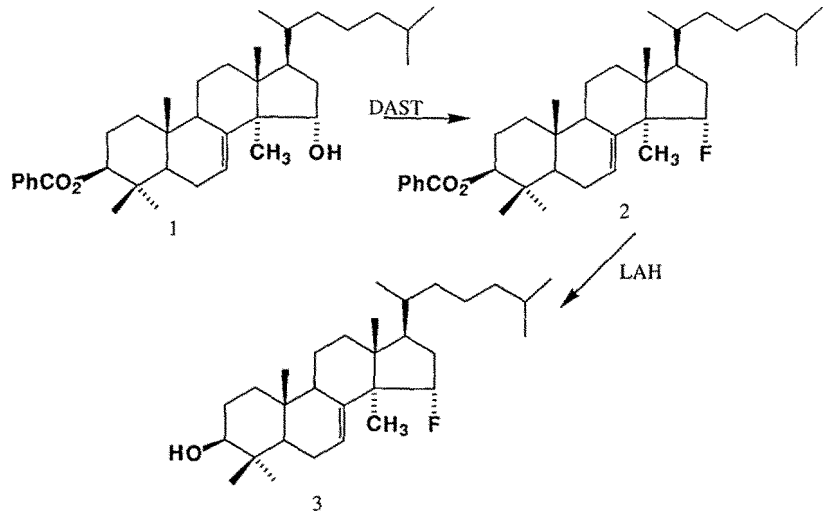
Physical data (**2**): $[\alpha]^{25} = +45.9 \pm 2.0°$ (c = 1.06, $CHCl_3$); m.p. = 226–227 °C (powder); R_F = 0.50 (5% ethyl acetate in hexane); 0.15 (25% toluene in hexane); NMR (300 MHz, $CDCl_3$): 8.06 (d, J = 7.4 Hz, 2H, phenyl), 7.55 (m, 1H, phenyl), 7.45 (m, 2H, phenyl), 5.51 (m, 1H, 7-CH), 5.05 (ddd, J = 56.6, 9.2, 5.6 Hz, 1H, 15-CHF), 4.78 (dd, J = 9.4, 5.6 Hz, 1H, 3-CHO), 2.18–1.27 (m, 23H), 1.15 (s, 3H, 31-CH_3), 1.10 (d, J = 3.6 Hz, 3H, 32-CH_3), 0.95 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.87 (unresolved d, 9H, 21, 26, 27-CH_3), 0.70 (s, 3H, 18-CH_3); IR (KBr, cm^{-1}): 2960 (m), 2930 (m, CH saturated), 1705 (s, C=O), 1460 (m), 1450 (m), 1390 (m), 1380 (m), 1365 (m), 1280 (s, C—O), 1120 (m, C—F), 1035 (m), 1025 (m); MS (EI): 550 (20% M^+), 413 (100%, M—$C_8H_9O_2$); high resolution mass spectroscopy for $C_{37}H_{55}O_2F$ (M^+): calculated 550.4186, found 550.4163.

Preparation of 15α-Fluorolanost-7-en-3β-ol (**3**)—Lithium aluminum hydride (290 mg, 7.6 mmol) was added slowly to a solution of 3β-benzoyloxy-15α-fluorolanost-7-ene (2294 mg, 0.534 mmol) in diethyl ether (40 ml) and tetrahydrofuran (10 ml) at O °C under nitrogen. The reaction was stirred for 10 min, then quenched with the addition of sodium sulfate decahydrate (1 g). The mixture was diluted with diethyl ether (50 ml) followed by ethyl acetate (20 ml). The resultant solution was filtered though a sintered glass funnel and the solvents removed *in vacuo*. The resulting solid was crystallized from isopropanol to give 230 mg (96% yield) of 15α-fluorolanost-7-en-3β-ol (**3**). The crystals had a m.p. = 159–160 °C, and the NMR showed co-crystallization with isopropanol (2:1, **3**:isopropanol). The crystals were then dissolved in benzene and the solvent removed to give an amorphous solid.

Physical Data (**3**): $[\alpha]^{25} = +24.6 \pm 4.0°$ (c = 0.5, $CHC1_3$); m.p. = 172.5–173 °C (powder); R_F = 0.18 (1:2:7, ethyl acetate:toluene:hexane); NMR (300 MHz, $CDCl_3$): 5.50 (m, 1H, 7-CH), 5.05 (ddd, J = 56.6, 9.2, 5.6 Hz, 1H, 15β-CHF), 3.27 (dd, J = 10.6, 4.9 Hz, 1H, 3-CHO), 2.18–1.27 (m, 23H), 1.10 (d, J = 3.6 Hz, 32-CH_3), 1.01 (s, ^{3}H, CH_3), 0.92–0.87 (m, 12H, methyls), 0.71 (s, 3H, 18-CH_3); ^{19}F NMR (188.2 MHz, $CDCl_3$): −192.9 (ddq, J = 56.6, 27.5, 3.6 Hz 15-CHF); IR ($CHC1_3$, cm^{-1}: 3620 (w, OH), 2960 (s), 2870 (m, CH sat), 1467 (m), 1448 (m), 1384 (m), 1367 (m), 1090 (w, CF), 1028 (m), 995 (m), 670 (m); MS (EI): 446 (65%, M^+), 431 (40%, $M^+ - CH_3$), 413 (50%, $M^+ - CH_3, -H_2O$), 306 (100%, $M^+ - C_9H_{16}O$); high resolution mass spectroscopy for $C_{30}H_{51}OF$ (M^+): calculated 446,3924, found 446.3917; EA for $C_{30}H_{51}OF$: calculated, C 80.66%, H 11.51%, F 4.25%; found, C 80.83%, H 11.29%, F 4.11%.

Synthesis of lanost-7-en-3β-ol used in these studies was performed as described by Woodward *et al.* (26).

Other Materials—All reagents used for lanosterol 14α-methyl demethylase and HMG-CoA reductase activity determinations were as described previously (13, 16). L-[^{35}S]Methionine (1120 Ci/mmol was from Du Pont-New England Nuclear. Guanidine isothiocyanate, CsCl, and oligo(dT)-cellulose Type 2 and Type 3 were from GIBCO. Agarose was from Bio-Rad. Nitrocellulose was obtained from



SCHEME II. **Synthetic route to 15α-fluorolanost-7-en-3β-ol.** Structures shown refer to those given in the text and identified by bolded numerals.

Schleicher and Schuell, and x-ray film was from Kodak. Anti-HMG-CoA reductase IgG was a generous gift of Dr. Gene Ness, University of Southern Florida. All other reagents were the best grade commercially available.

RESULTS

Characterization of 15α-Fluorolanost-7-en-3β-ol—The 15α-fluoro stereochemical assignment was critical for further studies with the 15α-fluorolanostenol in our enzymic and cell biological studies. This assignment was based upon NMR studies. The fluorine-proton coupling constant of 3.6 Hz which we observed between the C32-methyl and the fluorine groups is consistent with the cis orientation as shown in Scheme I (31). However, these data alone were insufficient for proper stereochemical assignment. Additional evidence was also provided by a series of nuclear Overhauser effect (NOE) studies. Previous NOE experiments conducted on 3β-benzoyloxylanost-7-en-15α-ol (27) gave an NOE between the 15β-hydrogen and the C18-methyl group. Conversely, the 3β-benzoyloxylanost-7-en-15β-ol provided an observed NOE between the 15α-hydrogen and the 32 methyl. In the case of the only observed fluorinated steroid from the diethylamino sulfur trifluoride reaction, we observe an NOE between the 15-hydrogen and the 18-methyl group. By analogy to the above cited experiments, these NOE results, along with the fluorine-proton coupling constants, allowed us to assigned the fluorinated steroid structure as the desired 3β-benzoyloxy-15α-fluorolanost-7-ene (**2**).

Metabolic Characteristics of 15α-Fluorolanost-7-en-3β-ol with Hepatic Microsomes—Once proper stereochemical assignments had been made, our initial studies with the 15α-fluorosterol were designed to assess its metabolic behavior with hepatic microsomes. Previously, we have shown that lanostenols with a Δ^7 double bond are poorly metabolized by the lanosterol demethylase despite having reasonable affinity for the enzyme (32). This poor metabolic characteristic coupled with the inherent difficulty in detecting oxylanostenols made formation and detection of the 15α-fluoro-3β-hydroxylanost-7-en-32-aldehyde a difficult task. Our efforts, therefore, did not focus upon detailed kinetics of formation, but rather upon simple demonstration of formation with more extensive characterization of the 15α-fluorolanosterol by inhibition kinetics toward the lanosterol 14α-methyl demethylase.

Shown in Fig. 1 is the GC/MS chromatogram of reaction products generated during incubation of 15α-fluorolanost-7-en-3β-ol with hepatic microsomes. The peak corresponding to

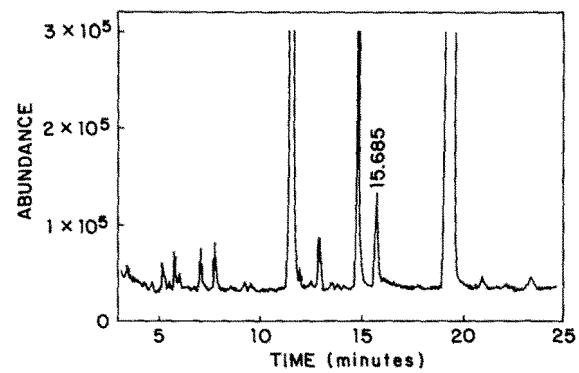


FIG. 1. **GC/MS chromatogram of reaction products of 15α-fluorolanost-7-en-3β-ol with hepatic microsomes.** The 15α-fluorolanostenol (250 μM) was incubated with hepatic microsomes (2.0 mg) for 1 h at 37 °C, at which time the assay was stopped by the addition of 15% KOH in 95% methanol. Following saponification and extraction, sterols were prepared for GC/MS analysis by reduction and TMS derivatization as detailed under "Methods." Chromatographic peaks identified by retention time are: TMS-cholesterol, 11.5 min; cholesterol, 14.8 min; diTMS-15α-fluorolanost-7-en-3β,32-diol, 15.7 min; and TMS-15α-fluorolanost-7-en-3β-ol, 19.0 min.

a retention time of 15.7 min represents the derivatized 15α-fluorolanostenol-32-aldehyde obtained through metabolism of the parent substrate seen at a retention time of 19.0 min. No metabolite was seen when incubations were done anaerobically or in the absence of NADPH (data not shown). As mentioned above, the lability of oxylanostenols to the gas chromatographic conditions employed during analysis required extensive derivatization prior to GC/MS identification (see "Methods"). Thus, the following fragmentation ions were observed in the mass spectrum of the reduced aldehyde TMS derivative: 571 (10%; $M^+ - F - CH_3$), 516 (12%; $M^+ -$ HOTMS), 484 (95%; $M^+ - F - CH_2OTMS$), 413 (20%; $M^+ -$ HOTMS $- CH_2OTMS$), 394(18%, $M^+ - C_9H_{15}OTMS$, 393 (18%; $M^+ -$ HOTMS $- CH_2OTMS - HF$) and 379 (46%; $M^+ - CH_3 - C_9H_{15}OTMS$). The absence of a molecular ion of 606 is consistent with the reported behavior of the lanostenol 14α-aldehyde in this GC/MS system (21). The loss of HF and F in the metabolite spectrum are also consistent with the same type losses in the parent structure which shows: 518 (26%; M^+), 428 (30%; $M^+ -$ HOTMS), 413 (88%; $M^+ -$ HOTMS $- CH_3$), 394 (10%; $M^+ - F -$ HOTMS $- CH_3$), 393 (10%; $M^+ - HF -$ HOTMS $- CH_3$). GC/MS analysis of reaction mixtures which were not reduced prior to derivati-

zation revealed only modest amounts of detectable 14α-alcohol (Table I). This finding indicates that the 14α-alcohol is present in the reaction mixture in much smaller amounts compared with the 14α-aldehyde, again consistent with previous metabolic studies employing dihydrolanosterol (21). Evidence for metabolism of the 15α-fluorolanosterol to the 32-formyloxy intermediate was not obtained in these studies. We attribute this lack of ability to generate the formyloxy intermediate to the inherent low rate of metabolism of the Δ^7 lanosterol series (32) and the inability to generate the formyloxy intermediate when using lanosterol as substrate due to the kinetic properties of the 14α-demethylase enzyme (33).

Further evaluation of the 15α-fluorolanostenol as a substrate for the lanosterol 14α-methyl demethylase was based upon inhibition kinetics employing 24,25-dihydrolanosterol as substrate with a cell-free preparation of the demethylase enzyme. The double-reciprocal plot of demethylase activity in the presence of several concentrations of the 15α-fluorolanost-7-en-3β-ol is shown in Fig. 2. The data clearly demonstrate that the sterol is a competitive inhibitor of the demethylase with respect to dihydrolanosterol showing a K_i = 315 μM. These data are consistent with the observation that the 15α-fluorosterol inhibits the conversion of C_{30} sterols to C_{27} sterols in cultured CHO cells (50% inhibition at a concentration of 5 μM in the culture medium, data not shown). Thus, the notion that the 15α-fluorolanostenol is a substrate for the lanosterol 14α-methyl demethylase is confirmed by kinetic studies as well as by metabolic conversion.

The Effect of 15α-Fluorolanost-7-en-3β-ol upon HMG-CoA Reductase Activity in Chinese Hamster Ovary Cells—The next series of experiments with the fluorinated sterol were conducted in CHO cells. Our interest was in the ability of the compound to modulate HMG-CoA reductase activity through an oxysterol-dependent mechanism. As shown in Fig. 3, the 15α-fluorolanosterol shows a concentration-dependent suppression of HMG-CoA reductase activity. This is a unique and specific property for the 15α-fluoro-substituted lanostenol, since lanost-7-en-3β-ol, the non-fluorinated analogue, is without effect upon HMG-CoA reductase under identical conditions (Fig. 3). The nature of the suppression is consistent with an oxysterol-type mechanism since the decrease in HMG-CoA reductase activity is accompanied by a decrease in the amount of immunoreactive reductase protein as shown in Fig. 4. Confirmation that 15α-fluorinated lanostenol required metabolism by the lanosterol 14α-methyl demethylase to suppress HMG-CoA reductase activity in these cell experiments was obtained through investigations employing the lanosterol 14α-methyl demethylase-deficient mutant AR45 (18). Treatment of AR45 cells with the fluorinated sterol does not

TABLE I

GC/MS characterization of metabolites derived from Lanost-7-en-3β-ol and 15α-fluorolanost-7-en-3β-ol incubated with hepatic microsomes

Incubation of sterols with hepatic microsomes was performed under standard lanosterol demethylase conditions for 1 h with the following exceptions: 250 μM sterol, 5.0 mg of Triton WR-1339, and 10% dimethyl sulfoxide. Nonsaponifiable sterols were extracted as described previously (16) and were split into two equal portions for derivatization and analysis by the schemes outlined under "Methods." This differential derivatization procedure allows for quantitation of both lanosterol 14α-alcohol and 14α-aldehyde metabolities, as well as demethylated diene sterol.

Substrate	Product	Total sterol
		%
Lanost-7-en-3β-ol[a]		91.3
	Lanost-7-ene-3β,32-diol	0.8
	3β-Hydroxylanost-7-en-32-al	4.9
	4,4-Dimethylcholesta-7,14-dien-3β-ol	3.0
15α-Fluoro-lanost-7-en-3β-ol[b]		97.1
	15α-Fluoro-lanost-7-ene-3β,32-diol	0.6
	3β-Hydroxy-15 alpha-fluoro-lanost-7-en-32-al	2.3

[a] Conversion rate = 0.09 nmol/min/mg.
[b] Conversion rate = 0.03 nmol/min/mg.

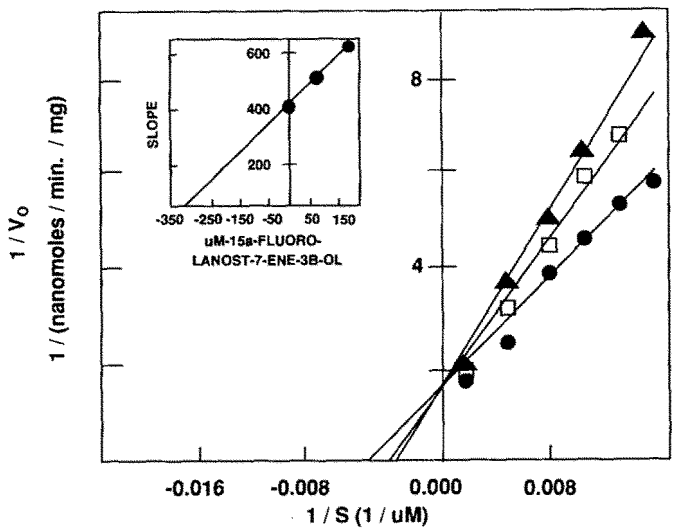


FIG. 2. **Kinetics of 15α-fluorolanost-7-en-3β-ol inhibition of lanosterol 14α-methyl demethylase activity.** Lanosterol 14α-methyl demethylase was assayed using [24,25-$^3H_2$]24,25-dihydrolanosterol as substrate in the absence (●) or presence of 100 μM (□) or 200 μM (▲) 15α-fluorolanost-7-en-3β-ol. Slopes of the reciprocal plots of the kinetic data were determined and plotted against inhibitor concentration (*inset*) to determine the inhibition constant K_i = 315 μM. Values plotted are the mean of duplicate determinations which varied by less than 5%.

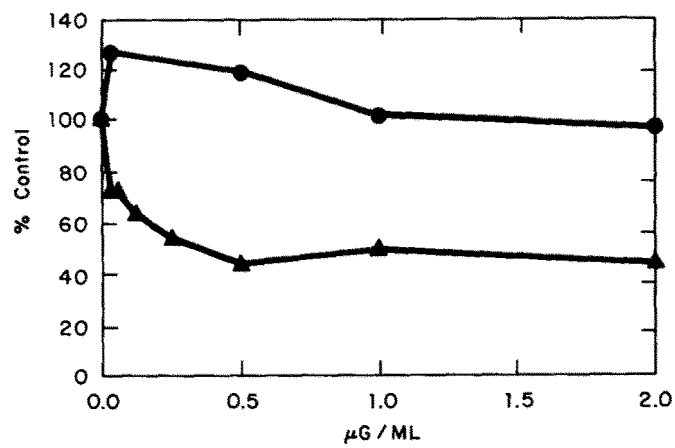


FIG. 3. **Suppression of HMG-CoA reductase activity in CHO cells treated with 15α-fluorolanost-7-en-3β-ol.** Chinese hamster ovary cells were treated with increasing concentration of either 15α-fluorolanost-7-en-3β-ol or lanost-7-en-3β-ol as described under "Methods." After 6-h exposure to the sterol, cells where processed for HMG-CoA reductase activity determinations employing the digitonin cell permeabilization assay (22). Results represent the average of duplicate determinations on replicate dishes. *Closed circles*, lanost-7-en-3β-ol; *closed triangles*, 15α-fluorolanost-7-en-3β-ol. Control (100%) activity = 479 pmol/min/mg. Standard errors were less than 5%.

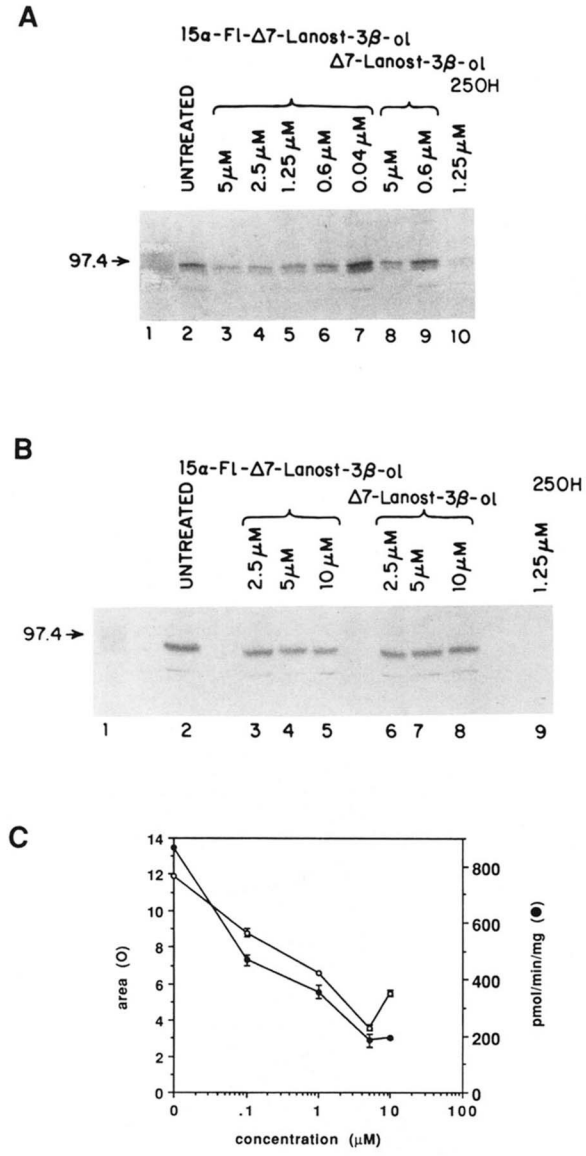


FIG. 4. **Immunoblot analysis of HMG-CoA reductase protein in sterol-treated cells.** Chinese hamster ovary cells were treated with either lanost-7-en-3β-ol or 15α-fluorolanost-7-en-3β-ol as described in the legend to Fig. 3. *A* and *B*, cells were harvested by scraping into sonication buffer, sonicated, and cell pellets isolated by centrifugation. Cell pellets were suspended in SDS-polyacrylamide gel electrophoresis buffer and 10 μg was loaded per well for separation and immunoblot analysis with anti-HMG-CoA reductase antibody. *A*, wild-type CHO cells. *B*, lanosterol 14α-methyl demethylase-deficient mutant, AR45, cells. *C*, immunoblots from CHO cells treated with 15α-fluorolanost-7-en-3β-ol were quantitated by scanning with a laser densitometer. The amount of immunoreactive protein is represented as the area under the absorbency curve. The average and range of values from replicate dishes are shown. Parallel cultures were harvested for determination of HMG-CoA reductase activity in cell sonicates as described under "Methods." Data for enzyme activity represent the means and standard errors of four samples. ●, enzyme activity; ○, immunoreactive protein.

produce a decrease in immunoreactive HMG-CoA reductase protein (Fig. 4*B*) nor does it inhibit reductase activity (control = 471 ± 30 pmol/min/mg; sterol = 539 ± 25 pmol/min/mg; n = 3). The lack of effect is not due to a nonresponsiveness of the AR45 cells to oxysterols, since suppression of HMG-CoA reductase is still observed when AR45 cells are treated with 25-hydroxycholesterol. Attempts to isolate the metabolic product of the 15α-fluorolanosterol from wild-type CHO cultures have thus far been unsuccessful.

Evidence for a Post-transcriptional Mechanism of HMG-CoA Reductase Regulation by 15α-Fluorolanost-7-en-3β-ol—To further characterize the suppression of HMG-CoA reductase caused by the 15α-fluorinated sterol, we measured the amount of reductase mRNA in treated and nontreated CHO cells. It was anticipated that the primary effect of the 15α-fluorosterol would be to change the level of reductase mRNA in a manner consistent with a transcriptional control process analogously to other sterols (3, 4). The effect with the 15α-fluorolanostenol upon reductase mRNA, however, was opposite to this expectation. The results of a representative experiment are shown in Fig. 5 and Table II. Chinese hamster ovary cells treated with the compound actually showed an increase in the amount of reductase mRNA compared with controls. In four independent experiments, the increase amounted to a 2-fold induction in reductase mRNA over a 16-h period. At the same time that the mRNA increase was observed, the level of reductase protein and activity decreased to 16% of control levels (Table III). This effect appears to be unique for the fluorinated sterol since 25-hydroxycholesterol decreased the amount of reductase mRNA, protein, and activity in agreement with published results (3, 4).

To further characterize the effects of the 15α-fluoro analogue on HMG-CoA reductase gene expression, the rates of reductase synthesis and degradation were measured. Synthesis of HMG-CoA reductase in CHO cells was measured by monitoring the incorporation of [^{35}S]methionine into immunoprecipitable protein in the presence or absence of sterol. The results of a representative experiment are shown in Fig. 6. Incubation of cells with the 15α-fluorolanostenol decreased the rate of synthesis of reductase (Fig. 6*B*) in the absence of any affect on total protein synthesis as assayed by the incorporation of [^{35}S]methionine into acid-perceptible protein (Fig. 6*A*). Table III, which summarizes the results of four independent experiments, show that the 15-fluorolanostenol inhibits HMG-CoA reductase synthesis without a corresponding decrease in mRNA levels. These observations suggest that in contrast to the transcriptional regulation of HMG-CoA reductase reported for other oxysterols, regulation by the 15-fluorolanostenol reduces the translational efficiency of reductase mRNA.

The effects of 15α-fluorolanost-7-en-β-ol on the half-life of HMG-CoA reductase were determined and results from a representative experiment are shown in Fig. 6*C*. Cells were

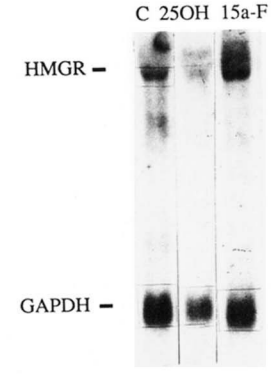


FIG. 5. **Autoradiogram of Northern blot analysis of poly(A$^+$) RNA in sterol-treated CHO cells.** Northern blot analysis of poly(A$^+$) RNA was performed as described under "Methods." Hybridizations were performed using nick-translated cDNA probes to HMG-CoA reductase (*HMGR*) and glutaraldehyde phosphate dehydrogenase (*GAPDH*). A representative autoradiogram is shown. *C*, ethanol control; *25OH*, 25-hydroxycholesterol (2.3 μM); *15α-F*, 15α-fluorolanost-7-en-3β-ol (5 μM).

TABLE II

Effect of 25-hydroxycholesterol and 15α-fluorolanost-7-en-3β-ol upon HMG-CoA reductase activity and mRNA levels in treated CHO cells

CHO cells were treated with 25-hydroxycholesterol or the 15α-fluorolanosterol at 2.5 μM for 16 h. Cells were harvested for HMG-CoA reductase activity measurements and isolation of poly(A⁺) RNA. Activity and mRNA levels were determined as outlined under "Methods" on pooled triplicate cultures. Quantitation of mRNA was done by cutting bands identified by autoradiography (see Fig. 5) and liquid scintillation counting. HMGR, HMG-CoA reductase.

Condition	HMGR activity[a]	HMGR mRNA	GAPDH mRNA	mRNA ratio (HMGR/GAPDH)
	pmol/min/mg		*dpm*	
Exp. 1				
Media	668 ± 38	266	229	1.16 (100)[b]
25-Hydroxycholesterol	102 ± 2	145	211	0.69 (59)
15α-Fluorolanost-7-en-3β-ol	108 ± 8	462	189	2.44 (210)
			Actin mRNA	mRNA ratio (HMGR/Actin)
Exp. II				
Media		856	375	2.28 (100)
25-Hydroxycholesterol		376	285	1.32 (58)
15α-Fluorolanost-7-en-3β-ol		1947	387	5.03 (221)

[a] Average of triplicate assays ± S.D.

[b] Numbers in parentheses are percent media control value.

TABLE III

Effect of 25-hydroxycholesterol and 15α-fluorolanost-7-en-3β-ol upon HMG-CoA reductase activity, mRNA, synthesis, and degradation in treated CHO cells

CHO cells were treated with 25-hydroxycholesterol (25-OH) or 15α-fluorolanosterol (15α-F). Enzyme activity, mRNA levels, and synthesis rates were determined as outlined under "Methods" and values are expressed as a percent of control. Enzyme half-life was determined as described in Fig. 6. All values are means ± S.E., with the number of independent experiments given in parentheses.

Sterol	Activity	Protein	mRNA	Synthesis	Degradation, $t_{1/2}$
		% control			*h*
Control	100	100	100	100	3.1 ± 0.2 (9)
25-OH	17 ± 3 (4)	15 ± 5 (3)	25 ± 5 (5)	20 ± 4 (2)	1.3 ± 0.3 (3)
15α-F	45 ± 5 (7)	46 ± 1 (2)	195 ± 21 (4)	65 ± 5 (4)	3.2 ± 0.3 (5)

either pulse-labeled with [^{35}S]methionine and chased with excess unlabeled methionine in the presence or absence of sterol or pretreated for 16 h with 15α-fluorolanost-7-en-β-ol and then labeled and chased in the presence of sterol. Although pretreatment with sterol reduced the incorporation of [^{35}S]methionine into reductase during the 2-h pulse ($t = 0$), the 15α-fluorolanostenol had no effect on the half-life of the enzyme. The average rate of degradation of the newly synthesized reductase determined in five separate experiments was not significantly different from that seen in control cells (Table III). It has been suggested that sterol regulation of reductase degradation is indirect and mediated by its effects on enzyme activity and mevalonate availability (7, 34). Thus the lack of effect of 15α-fluorolanostenol on HMG-CoA reductase degradation might be related to its relative lack of potency as an inhibitor or reductase (Table II). To test this possibility, 25-hydroxycholesterol, a potent transcriptional regulator of HMG-CoA reductase (Ref. 3 and Fig. 4), was added to cells at a concentration of 250 nM. This resulted in a 45% decrease in HMG-CoA reductase activity after 6 h, a value similar to that seen when cells are treated for 16 h with 10 μM 15α-fluorolanostenol. Under these conditions, 25-hydroxycholesterol increased the rate of degradation of HMG-CoA reductase 3.5-fold (data not shown). The inability of 15α-fluorolanostenol to accelerate reductase degradation is thus not a result of its more moderate inhibition of reductase activity. The data for oxysterol effects on HMG-CoA reductase activity, mRNA, synthesis, and degradation are summarized in Table III. These results suggest that 15α-fluorolanostenol, in contrast to 25-hydroxycholesterol, is a post-transcriptional regulator of HMG-CoA reductase which acts solely at the level of translation.

DISCUSSION

The results presented in the current study characterize the metabolic behavior of 15α-fluorolanost-7-en-3β-ol, a synthetic analogue of the natural substrate for the lanosterol 14α-methyl demethylase enzyme. We have shown that the 15α-fluorolanostenol functions as a substrate for the lanosterol 14α-methyl demethylase, as well as serves as a good competitive inhibitor of the enzyme. Our results substantiate and extend previous findings that 15-fluoro-substituted lanostenols block cholesterol biosynthesis from lanosterol (30). By virtue of the 15α-fluoro substitution, it has been possible to block complete metabolism of this lanosterol analogue to demethylated sterol which results in accumulation of oxygenated sterol under normal metabolic conditions. These findings indicate that 15α-fluoro substitution in the lanostenol molecule is a modest modification which functions only to impede metabolic conversion to end product cholesterol, but not other properties of the lanostenol molecule. Thus, the ability to generate a stable oxylanosterol analogue through metabolism of the 15α-fluorosterol as described in this report provides us with another tool to study the mechanism of oxylanosterol regulation of cholesterol synthesis and HMG-CoA reductase activity.

Experiments in cultured cells with the 15α-fluorolanostenol have demonstrated that the 15α-fluorolanost-7-en-3β-ol is an active suppressor of HMG-CoA reductase activity under conditions when metabolism to an oxylanosterol analogue is permissive. We observe suppression of the HMG-CoA reductase activity only in wild-type CHO cells and not in the lanosterol 14α-methyl demethylase-deficient mutant, AR45. These results strongly suggest that metabolism of the 15α-fluorolanostenol by the lanosterol 14α-methyl demethylase enzyme is required for suppressor activity. Additionally, the decrease in enzyme activity is accompanied by a decrease immunoreactive protein which is consistent with previous reports on oxylanosterol regulation of HMG-CoA reductase activity (14).

Mechanistic studies with sterols, and in particular cholesterol and 25-hydroxycholesterol, have shown that sterol-de-

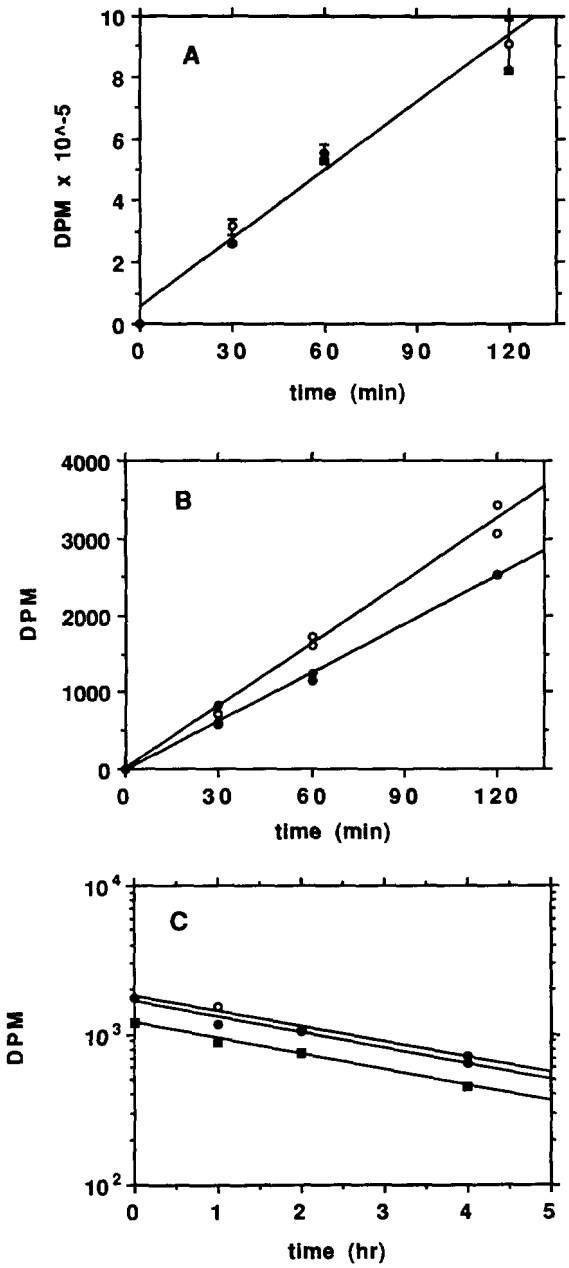


FIG. 6. **Effects of 15α-fluorolanost-7-en-3β-ol on HMG-CoA reductase synthesis and degradation.** *A* and *B*, CHO cells were labeled with [^{35}S]methionine for the indicated times in the presence of (●) or absence (○) of sterols. Incorporation of [^{35}S] methionine into total acid-perceptible proteins and immunoprecipitable HMG-CoA reductase was determined as described under "Methods." *A*, acid-precipitable radioactivity. Data represent the average and standard error of duplicate determinations on replicate dishes. *B*, HMG-CoA reductase. Data represent disintegrations/min eluted from gel slices, corrected for nonspecific radioactivity. Curves were fitted by linear least squares analysis (Cricket Graph, Microsoft) ($R^2 > 0.9$). *C*, CHO cells were pulse-labeled for 2 h with [^{35}S]methionine and chased in the presence of unlabeled methionine for the indicated times. Cells were harvested and radiolabeled HMG-CoA reductase was quantitated as described under "Methods." Curves were fitted by exponential least squares analysis ($R^2 > 0.97$). ○, ethanol control; ●, 15α-fluorolanost-7-en-3β-ol was added at the beginning of the chase; ■, cells were treated with 15α-fluorolanost-7-en-3β-ol for 16 h prior to the addition of label.

pendent regulation of HMG-CoA reductase activity and expression is due to inhibition of transcription of the reductase gene (3). An octanucleotide cis-acting DNA sequence in the 5′-flanking region of the reductase gene appears to be responsible for sterol-dependent control of gene transcription and is thought to be the core binding site for a sterol-binding protein which represses transcription (35–37). It has also been demonstrated that regulation of reductase activity by sterols correlates with the ability of sterols to interact with a cytosolic oxysterol-binding protein (38). Viewed collectively, these data support the conclusion that sterols regulate HMG-CoA reductase through transcriptional control mediated at least in part through the action of a common oxysterol binding protein. The data we have presented, however, would indicate that this general conclusion regarding sterols may not be valid. Our results show that the 15α-fluorooxylanosterol analogue generated *in situ* regulates reductase expression through a post-transcriptional process. The decrease in reductase activity caused by the sterol is not accompanied by a decrease in reductase mRNA, but rather an actual increase. Similar post-transcriptional control of HMG-CoA reductase expression by 24,25-epoxylanosterol has been described (39). When metabolism of this compound to the epoxycholesterol was blocked, it no longer inhibited reductase activity in a rat intestinal epithelial cell line. However, in both primary rat hepatocytes and CHO cells, the oxidolanosterol post-transcriptionally inhibited reductase synthesis and enhanced reductase degradation even when its demethylation was inhibited with ketoconazole (39). In contrast to all other sterols examined to date, treatment of CHO cells with the 15α-fluorolanostenol has no effect on the degradation rate of HMG-CoA reductase. The 15α-fluorooxylanostenol metabolite thus appears to be unique in the manner of its regulation of HMG-CoA reductase, inhibiting translation without affecting either transcription or enzyme degradation.

The present findings dictate that previous assumptions and data used to support the concept of a non-steroidal regulator of HMG-CoA reductase activity be re-evaluated. In particular, the basic premise that all sterols regulate HMG-CoA reductase through a 25-hydroxycholesterol mechanism is shown here to be false. Thus, experiments which employ 25-hydroxycholesterol to totally account for sterol regulation are subject to misinterpretation. For example, when 25-hydroxycholesterol is used in combination with mevalonic acid to regulate HMG-CoA reductase activity, the mevalonic acid effect cannot be assigned exclusively to a "non-steroidal" regulator. In fact, conditions which employ mevalonic acid to generate a non-steroidal regulator, either in mutant cells (6), or in combination experiments using 25-hydroxycholesterol plus compactin (7), favor synthesis of oxylanosterols (15). Of interest in this regard is the report that inhibition of HMG-CoA reductase by a 32-carboxylic acid derivative of lanosterol (SKF 104976) requires mevalonate (40), whereas inhibition by 24(*S*),25-oxidolanosterol does not (39). Additionally, Chin *et al.* (41) have shown that sterols can effect HMG-CoA reductase expression independently of transcriptional processes. Cohen and Griffioen (42) have also differentiated sterol metabolites of mevalonate whose effects upon reductase mRNA can or cannot be replaced by exogenous low density lipoprotein. Thus, the designation of the reductase translational regulator as non-steroidal should not be accepted without further proof of identity obtained through isolation and absolute characterization.

Our approach to understanding the mechanism of regulation of HMG-CoA reductase by endogenously generated oxysterols has focused on the lanosterol 14α-methyl demethylation cycle (13, 14, 16). Synthesis, chemical characterization, and metabolic studies with the novel substrate/inhibitor, 15α-fluorolanost-7-en-3β-ol, described in this report, extend our

previous findings in this general area. The novel metabolic behavior of the 15α-fluorolanostenol demonstrates the possibility that manipulation of the lanosterol demethylation cycle through targeted synthesis can lead to a novel means to control HMG-CoA reductase activity and cholesterol biosynthesis. Our activities are continuing in this area.

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