Inhibition of sterol biosynthesis in animal cells by 14α-hydroxymethyl sterols

George J. Schroepfer, Jr.,[1] Edward J. Parish, Robert A. Pascal, Jr.,[2] and Andrew A. Kandutsch[3]

Departments of Biochemistry and Chemistry, Rice University, Houston, TX 77001 and the Jackson Laboratory, Bar Harbor, ME 04609

Abstract The chemical syntheses of a number of 14α-hydroxymethyl sterols and 14α-hydroxymethyl-15α-hydroxysterols and their derivatives have been pursued to permit evaluation of their activity in the inhibition of sterol biosynthesis in animal cells in culture. Described herein are chemical syntheses of 7α,8α-epoxy-14α-methyl-5α-cholestan-3β,15α-diol, 14α-methyl-5α-cholestan-3β,7α,15α-triol, 3β,15α-diacetoxy-14α-methyl-5α-cholestan-7α-ol, 3β,15α-diacetoxy-7α,32-epoxy-14α-methyl-5α-cholestane, 14α-hydroxymethyl-5α-cholest-6-en-3β,15α-diol, 14α-hydroxymethyl-5α-cholest-7-en-3β,15α-diol, 7α,32-epoxy-14α-methyl-5α-cholestan-3β,15α-diol, 14α-hydroxymethyl-5α-cholest-6-en-3-one, 14α-hydroxymethyl-5α-cholest-7-en-3-one, and 14α-hydroxymethyl-5α-cholest-7-en-15α-ol-3-one. The effects of eight of the above compounds and of 14α-hydroxymethyl-5α-cholest-8-en-3β-ol, 14α-hydroxymethyl-5α-cholest-7-en-3β-ol, 14α-hydroxymethyl-5α-cholest-6-en-3β-ol, and 7α,32-epoxy-14α-methyl-5α-cholestan-3β-ol on the synthesis of digitonin-precipitable sterols and on levels of HMG-CoA reductase activity in L cells and in primary cultures of fetal mouse liver cells have been investigated. All of the 14α-hydroxymethyl sterols and 14α-hydroxymethyl-15α-hydroxysterols were found to be potent inhibitors of sterol synthesis and to reduce the levels of HMG-CoA reductase activity in these cells. Since hydroxylation of the 14α-methyl group of 14α-methyl sterol precursors of cholesterol can be considered as an obligatory step in the biosynthesis of cholesterol, the finding that 14α-hydroxymethyl sterols are potent inhibitors of cholesterol biosynthesis and cause a reduction in the levels of HMG-CoA reductase activity raises the possibility that oxygenated sterol precursors of cholesterol, such as 14α-hydroxymethyl sterols, may play an important role in the regulation of cholesterol synthesis and in the regulation of processes dependent upon mevalonate and sterol formation.—**Schroepfer, G. J., Jr., E. J. Parish, R. A. Pascal, Jr., and A. A. Kandutsch.** Inhibition of sterol biosynthesis in animal cells by 14α-hydroxymethyl sterols. *J. Lipid Res.* 1980. **21:** 571–584.

Supplementary key words 14α-hydroxymethyl-15α-hydroxysterols · HMG-CoA reductase · fetal mouse liver cells

Oxygenated metabolites of cholesterol have been shown to be potent inhibitors of cholesterol biosynthesis (1–4). This inhibition of sterol synthesis has been demonstrated to be associated with a suppression of the activity of the key regulatory enzyme HMG-CoA reductase. Some of these inhibiting sterols are either established or possible intermediates in the conversion of cholesterol to bile acids or to steroid hormones and they may regulate the biological formation of cholesterol required for these purposes.

The purpose of the present study was to explore the possibility that oxygenated precursors of cholesterol might also serve as potential regulators of sterol synthesis. In particular we have directed our attention to the possible inhibitory action of 14α-hydroxymethyl sterols on sterol biosynthesis. It has generally been considered that the first step in the enzymatic removal of each of the "extra" methyl groups of lanosterol is via an initial oxidation to yield the corresponding hydroxymethyl derivative (5–8). In the case of the removal of the 14α-methyl group (carbon atom 32), the initial enzymatic reaction would yield a 14α-hydroxymethyl derivative. In this regard it is important to note that a number of 14α-hydroxymethyl Δ^8- and Δ^7-sterols have been shown to be convertible to cholesterol upon incubation with the 10,000 *g* supernatant fraction of rat liver homogenates (9–12). We now wish to report that a number of 14α-hydroxymethyl sterols, with and without a hydroxyl function of the C-15 position, are potent inhibitors of sterol synthesis in L cells and in primary cultures of fetal mouse liver cells and cause a reduction

Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; IR, infrared; TMS, trimethylsilyl; MPLC, medium pressure liquid chromatography; MS, mass spectral.

[1] To whom inquiries should be directed at Rice University.

[2] Recipient of predoctoral fellowship from the National Science Foundation.

[3] To whom inquiries should be directed at the Jackson Laboratory.

of the level of HMG-CoA reductase activity in these cells.[4] These findings are of considerable potential importance since, in view of our current understanding of the reactions involved in cholesterol biosynthesis, 14α-hydroxymethyl sterols should be formed in all cells which have the capacity for the de novo synthesis of cholesterol. Moreover, since a cycle of cholesterol biosynthesis appears to be required for replication of cells (including cells which do not form oxygenated metabolites of cholesterol) (4 and references cited therein), it is possible that 14α-hydroxymethyl sterol precursors of cholesterol regulate HMG-CoA reductase and sterol formation during the cell cycle.

A preliminary account of a portion of this research has been published (13).

EXPERIMENTAL PROCEDURES

General

Melting points were recorded in sealed evacuated capillary tubes using a Thomas Hoover melting point apparatus. Infrared (IR) spectra were recorded on a Beckman IR-9 spectrometer using KBr pellets. Thin-layer chromatographic (TLC) analyses were made on plates of silica gel G (E. Merck, Darmstadt). Components on the plates were visualized after spraying with molybdic acid (14). Gas–liquid chromatographic (GLC) analyses were made using a Hewlett-Packard Model 402 unit equipped with dual flame ionization detectors. The columns (6 ft × 0.25 in, o.d.) were packed on 3% OV-1 or 3% OV-17 on Gas-Chrom Q (100/120 mesh). Medium pressure liquid chromatography (MPLC) was performed using columns of silica gel (0.032–0.063 mm; Woelm). Low resolution mass spectral (MS) analyses were made using an LKB-9000S spectrometer under operating conditions described previously (15). High resolution mass spectral analyses were made on a Varian CH-5 spectrometer (courtesy of Professor C. C. Sweeley). Trimethylsilyl (TMS) ether derivatives of the sterols (~0.1 mg) were prepared by treatment with a mixture of hexamethyldisilazane, trimethylchlorosilane, and pyridine (0.1 ml; 13:8:10) at 50° for 10 min. Nuclear magnetic resonance (NMR) spectra were recorded on a Perkin-Elmer HR-12 spectrometer at 60 MHz or on a Varian EM-390 spectrometer at 90 MHz using tetramethylsilane as an internal standard. Peaks are reported as ppm (δ) downfield from the tetramethylsilane standard. Unless otherwise specified, $CDCl_3$ was used as the solvent. 14α-Methyl-5α-cholest-7-en-3β,15α-diol (XII) was prepared as described previously (16, 17). The syntheses of 14α-hydroxymethyl-5α-cholest-8-en-3β-ol (VI), 14α-hydroxymethyl-5α-cholest-6-en-3β-ol (VII), 14α-hydroxymethyl-5α-cholest-7-en-3β-ol (VIII), and of 7α,32-epoxy-14α-methyl-5α-cholestan-3β-ol (XXII) have been described previously (13, 18). Pyridine hydrochloride was prepared by passage of anhydrous gaseous HCl through ether (200 ml) containing pyridine (25%) for 10 min. The resulting precipitate was collected by filtration, washed with anhydrous ether, and dried in a vacuum dessicator. Cholesterol oxidase (components 1 and 3 of the Cholesterol Auto Test) was purchased from Biodynamics (BMC Division), Boehringer Mannheim (Indianapolis, IN).

Cell cultures

Primary cultures of fetal mouse liver cells and mouse L cell cultures were grown as monolayers in serum-free, chemically defined media in 75 cm^2 culture flasks as previously described (1, 2, 22).

Assays for suppression of HMG-CoA reductase activity and inhibition of sterol synthesis

Methods for the measurement of DNA and protein were described previously (1). Procedures for assaying rates of conversion of [1-^{14}C]acetate into digitonin-precipitable sterols, fatty acids and CO_2 were the same as described previously (1, 2) except that following the incubation of the cultures with the labeled acetate, [1,2-^{3}H]cholesterol (40,000 dpm; 0.3 pmol; New England Nuclear Corp.) was added to each flask to permit estimation of [^{14}C]sterol recoveries. Studies of rates of acetate metabolism to fatty acids and, in some cases, to CO_2 were also made so as to detect any possible effects of the inhibitors of sterol synthesis on general metabolism. In the present series of tests, unless stated otherwise, the compounds examined showed no consistent effects on the rates of fatty acid or CO_2 production. Minor variations in rates of fatty acid formation and CO_2 production in experimental flasks as well as in control flasks were, therefore, considered to be due to technical error and to variations in the metabolic characteristics of individual cultures in a similarly prepared and similarly treated experimental group. In an effort to correct for the effects of these sources of variation upon estimates of inhibitory potency, concentrations required to inhibit sterol synthesis by 50% were estimated from plots of the ratio of [^{14}C]sterols to [^{14}C]fatty acids as a function of the concentration of the inhibitor (1).

[4] In addition, recent evidence has been obtained that Δ^8- and Δ^7-lanostenols with a 32-alcohol or aldehyde function suppress HMG-CoA reductase activity. (G. F. Gibbons, C. R. Pullinger, H. W. Chen, W. K. Cavenee, and A. A. Kandutsch. Submitted for publication.)

HMG-CoA reductase activity in the L cells and in the fetal mouse liver cells was assayed by a minor modification (23) of procedures described previously (1, 2).

Chemical synthesis of sterols

7α,8α-Epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XIV). *m*-Chloroperbenzoic acid (2.00 g; 85%) and $NaHCO_3$ (1.6 g) were added to a solution of XII (4.00 g; 9.62 mmol) in CH_2Cl_2 (275 ml) and the resulting mixture was stirred at 25°C for 36 hr. Ether was added and the solution was washed with 1N NaOH and water. The organic phase was dried over $MgSO_4$ and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XIV (3.87 g; 93% yield) whose purity was established as ~95% by analysis by TLC (solvent, 50% ethyl acetate in benzene). The compound melted at 140.0–141.5°C; IR, ν_{max} 3460, 1049, 980, and 513 cm^{-1}; NMR, 0.85 (s, 3H, C-19-CH_3), 0.88 (s, 3H, C-18-CH_3), 1.11 (s, 3H, C-32-CH_3), 3.55 (m, 2H, C-3-H and C-7-H), 4.15 (m, 1H, C-15-H); MS, 432 (M; 26%), 417 (M-CH_3; 7%), 414 (M-H_2O; 68%), 399 (M-CH_3-H_2O; 24%), 396 (M-H_2O-H_2O; 8%), 381 (M-CH_3-H_2O-H_2O; 70%), 363 (8%), 355 (10%), 320 (34%), 301 (M-H_2O-side chain; 100%), 283 (M-H_2O-H_2O-side chain; 12%), 277 (16%), 259 (15%), 223 (30%), high resolution MS, 432.3615 (calc. for $C_{28}H_{48}O_3$: 432.3603).

14α-Methyl-5α-cholestan-3β,7α,15α-triol (XV) and 14α-methyl-5α-cholest-7-en-3β,15α-diol (XII) from reduction of 7α,8α-epoxy-14α-methyl-5α-cholestan-3β,15α-diol with lithium-ethylamine. Lithium (2 g) was added to ethylamine (150 ml; freshly distilled and dried over Linde type 3A molecular sieves) and the mixture was stirred at 25°C under nitrogen until a dark blue color appeared, whereupon XIV (1.00 g; 2.31 mmol) was added. After stirring at 25°C for 75 min, CH_3OH was added dropwise to discharge the blue color. Additional CH_3OH was added until complete solution occurred. Water and ice were then added until all of the sterol had precipitated. The product was collected on a filter, washed with water, and dried. Analysis by TLC (solvent, 50% ethyl acetate in benzene) of the crude product (0.96 g) indicated two major components with R_f values of 0.06 and 0.42. This material was subjected to MPLC on a silica gel column (118 cm × 1.5 cm) using 20% benzene in ethyl acetate as the eluting solvent at a flow rate of 4.0 ml per min. Fractions 20 ml in volume were collected. The contents of fractions 9 through 17 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XII (0.36 g; 37% yield) melting at 192.5–193.5°C (literature: 192–193° (16, 17)) IR, ν_{max} 3420, 1670, 1055, and 631 cm^{-1}; NMR, 0.70 (s, 3H, C-18-CH_3), 0.81 (s, 3H, C-19-CH_3), 1.20 (s, 3H, C-32-CH_3), 3.70 (m, 1H, C-3-H), 4.20 (m, 1H, C-15-H), 5.40 (m, 1H, C-7-H); MS, 416 (M; 63%), 401 (M-CH_3; 30%), 398 (M-H_2O; 9%), 383 (M-CH_3-H_2O; 100%), 365 (M-CH_3-H_2O; 23%), 276 (10%), 261 (47%), 247 (7%), 231 (9%), 219 (12%), and 205 (30%); the product showed a single component on analysis by TLC in three solvent systems (10% ether in benzene, 35% ethyl acetate in $CHCl_3$, and 50% benzene in ethyl acetate) and a purity of ~99.5% on GLC (3% OV-1 and 3% OV-17; 270°C) with the identical chromatographic properties of an authentic sample.

The contents of fractions 41 through 110 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XV (0.53 g; 53% yield) melting at 200.5–201.5°C; IR, ν_{max} 3420, 1050, and 900 cm^{-1}; NMR, 0.81 (s, 3H, C-18-CH_3), 0.88 (s, 3H, C-19-CH_3), 1.12 (s, 3H, C-32-CH_3), 3.60 (m, 1H, C-3-H), 4.18 (m, 2H, C-7-H and C-15-H); MS, 434 (M; 1%), 416 (M-H_2O; 36%), 414 (4%), 401 (M-CH_3-H_2O; 23%), 398 (M-H_2O-H_2O; 19%), 383 (M-CH_3-H_2O-H_2O; 37%), 365(M-CH_3-H_2O-H_2O-H_2O; 10%), 320 (14%), 303 (M-H_2O-side chain; 13%), 285 (M-H_2O-H_2O-side chain, 38%), 276 (100%), 267 (M-H_2O-H_2O-H_2O-side chain; 11%), 261 (75%), and 207 (39%); high resolution MS, 434.3732 (calc. for $C_{28}H_{50}O_3$: 434.3744). The compound showed a single component on TLC in two solvent systems (50% benzene in ethyl acetate and 50% $CHCl_3$ in ethyl acetate) and a purity of ~98.5% on GLC of the *tris*-TMS derivative (3% OV-1 and 3% OV-17; 270°C).

3β,15α-Diacetoxy-14α-methyl-5α-cholestan-7α-ol (XVI). To XV (2.00 g; 4.60 mmol) in pyridine (150 ml), cooled to 0°C, was added cold (0°C) acetic anhydride (50 ml). The reaction mixture was maintained at 4°C for 24 hr with occasional stirring. Ice was added and the mixture was poured into ether (1,000 ml) and washed successively with water, cold 5% HCl, 5% $NaHCO_3$, and water. The organic extract was dried over $MgSO_4$ and the residue obtained upon evaporation of the solvent was subjected to MPLC on a silica gel column (118 cm × 1.5 cm) using 15% ethyl acetate in benzene as the eluting solvent at a flow rate of 4.0 ml per min. Fractions 20 ml in volume were collected. The contents of fractions 75 through 110 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XVI (2.00 g; 84% yield) which melted at 195.0–196.5°C; IR, ν_{max} 3550, 1730, 1260, and 1035 cm^{-1}; NMR, 0.81 (s, 3H, C-18-CH_3), 0.88 (s, 3H, C-19-CH_3), 1.23 (s, 3H, C-32-CH_3), 1.99 and 2.03 (s, 3H each, methyls of acetoxy functions), 3.90 (m, 1H, C-7-H), and 4.80 (m, 2H, C-3-H and C-15-H); MS, 500 (M-H_2O; 1%), 458 (M-CH_3COOH; 92%), 443 (M-CH_3-

CH_3COOH;30%), 440 (M-H_2O-CH_3COOH;12%), 425 (M-CH_3-H_2O-CH_3COOH; 19%), 398 (M-CH_3COOH-CH_3COOH; 10%), 383 (M-CH_3-CH_3COOH-CH_3-$COOH$;8%), 345 (M-CH_3COOH-side chain;20%), 327 (M-side chain-H_2O-CH_3COOH; 8%), 318 (100%), 303 (74%), 291 (24%), 289 (30%), 275 (21%), 272 (17%), 267(M-H_2O-CH_3COOH-CH_3COOH-side chain;15%), 261 (14%), 258 (17%), 249 (46%), and 236 (58%); MS of TMS ether derivative, 590 (M; 1%), 530 (M-CH_3COOH; 100%), 515 (M-CH_3-CH_3COOH; 9%), 500 (M-trimethylsilanol; 2%), 470 (M-CH_3COOH-CH_3COOH; 20%), 455 (M-CH_3-CH_3COOH-CH_3-$COOH$; 3%), 440 (M-CH_3COOH-trimethylsilanol; 23%), 425 (M-CH_3-CH_3COOH-trimethylsilanol; 10%), 417 (M-CH_3COOH-side chain; 3%), 398 (14%), 380 (M-CH_3COOH-CH_3COOH-trimethylsilanol; 14%), 365 (M-CH_3-CH_3COOH-CH_3COOH-trimethylsilanol; 14%), 327 (M-CH_3COOH-trimethylsilanol-side chain; 25%), 321 (22%), 267 (M-CH_3COOH-CH_3COOH-trimethylsilanol-side chain; 20%), high resolution MS, 590.4398 (calc. for $C_{35}H_{62}O_5{}^{28}Si$: 590.4371). The compound showed a single component on TLC in three solvent systems (35% $CHCl_3$ in ethyl acetate, 50% benzene in ethyl acetate, and 20% ether in benzene) and purity of ~98% on GLC of its TMS ether derivative (3% OV-1 and 3% OV-17; 270°).

3β,15α-Diacetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (XVII). Compound XVI (500 mg; 0.97 mmol) was dissolved in dry benzene (250 ml) and ~50 ml of the solvent was removed by distillation. Lead tetraacetate (2.10 g) was added and the resulting solution was heated under reflux for 24 hr. After the addition of a 20% KI solution (50 ml), a saturated solution of sodium thiosulfate was added until the yellow precipitate had dissolved. The mixture was thoroughly extracted with ether and the combined extracts were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The resulting residue was subjected to MPLC on a silica gel column (118 cm × 1.5 cm) using 10% ether in benzene as the eluting solvent at a flow rate of 4.0 ml per min. Fractions 20 ml in volume were collected. The contents of fractions 50 through 90 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XVII (320 mg; 74% yield) melting at 160–162°; IR, ν_{max} 1745, 1253, 1048, and 1030 cm^{-1}; NMR 0.82 (s, 3H, C-18-CH_3), 0.88 (s, 3H, C-19-CH_3), 1.99 and 2.03 (s, 3H each, methyls of acetoxy functions at C-3 and C-15), 4.00 (m, 3H, C-32-H_2 and C-7-H), 4.90 (m, 2H, C-3-H and C-15-H); MS, 516 (M; 1%), 456 (M-CH_3COOH; 100%), 441 (M-CH_3-CH_3COOH; 12%), 426 (M-CH_2OH-CH_3COOH; 39%), 425 (M-CH_2OH-CH_3COOH; 50%), 403 (M-side chain; 5%), 396 (M-CH_3COOH-CH_3COOH; 14%), 381 (M-CH_3-CH_3COOH-CH_3COOH; 5%), 371 (10%), 365 (M-CH_2OH-CH_3COOH-CH_3COOH; 11%), 343 (M-CH_3COOH-side chain; 33%), 315 (10%), 313 (M-CH_2O-CH_3COOH-side chain; 22%), 302 (90%), 283 (M-CH_3COOH-CH_3COOH-side chain; 11%), high resolution MS, 516.3836 (calc. for $C_{32}H_{52}O_5$: 516.3814). The compound showed a single component on TLC in three solvent systems (50% ethyl acetate in benzene, 35% $CHCl_3$ in ethyl acetate, and 20% ether in benzene) and a purity of ~98.6% on GLC (3% OV-1 and 3% OV-17; 270°C).

14α-Hydroxymethyl-5α-cholest-6-en-3β,15α-diol (XVIII), 14α-hydroxymethyl-5α-cholest-7-en-3β,15α-diol (XIX), and 7α,32-epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XX). Compound XVII (400 mg; 0.77 mmol) was heated under reflux with pyridine hydrochloride (800 mg) and acetic anhydride (20 ml) for 6 hr. The solution was poured into ice water, allowed to stand for 90 min, and thoroughly extracted with ether. The combined ether extracts were successively washed with cold 5% HCl, water, 5% Na_2CO_3, and water, dried over $MgSO_4$, and evaporated to dryness under reduced pressure. To the residue, dissolved in ether (50 ml), was added $LiAlH_4$ (500 mg). After standing for 2 hr at 25°C, the mixture was cooled to 0°C and ice was cautiously added to decompose the unreacted hydride. The resulting mixture was poured into a saturated solution of NH_4Cl and thoroughly extracted with ether containing CH_2Cl_2 (10%). The combined ether extracts were dried over $MgSO_4$ and evaporated to dryness under reduced pressure. Analysis by TLC (solvent, 50% benzene in ethyl acetate) indicated three major components with R_f values of 0.34, 0.17, and 0.08. The crude product was subjected to MPLC on a silica gel column (118 cm × 1.5 cm) using 40% ethyl acetate in benzene as the eluting solvent at a flow rate of 4.0 ml per min. Fractions 20 ml in volume were collected.

The contents of fractions 39 through 60, containing the least polar component, were pooled and, after evaporation of the solvent at 35°C under reduced pressure, gave a white crystalline solid which was washed with hexane to give XVIII (76 mg; 23% yield) which melted at 228.5–230.0°C; IR, ν_{max} 3400, 1650, 1077, 1038, 715, and 623 cm^{-1}; NMR, 0.81 (s, 3H, C-19-CH_3), 0.95 (s, 3H, C-18-CH_3), 3.24 (m, ~0.7H, H-bonded hydroxyl proton; disappeared on exchange with D_2O), 3.59 (m, ~1.2H, C-3-H and C-32-H; integrated for 1H after exchange with D_2O), 4.18 (m, ~2.8H, C-15-H and C-32-H_2; integrated for 3H after exchange with D_2O); 5.30 (d, 1H; J = 12 c.p.s.) and 6.10 (d, 1H, J = 12 c.p.s.); MS, 414 (M-H_2O; 100%), 401 (M-CH_2OH; 5%), 399 (M-CH_3-H_2O; 14%), 396 (M-H_2O-H_2O; 55%), 384 (M-CH_2O-

H_2O; 56%), 383 (M-H_2O-CH_2OH; 58%), 381 (M-CH_3-H_2O-H_2O; 51%), 369 (31%) 365 (M-H_2O-H_2O-CH_2OH; 31%), 363 (M-CH_3-H_2O-H_2O-H_2O; 54%), 355 (34%), 301 (M-H_2O-side chain; 28%), 295 (30%), 283 (M-H_2O-H_2O-side chain; 68%), 281 (34%) 271 (M-CH_2O-H_2O-side chain; 63%), 270 (M-H_2O-CH_2-OH-side chain; 10%), 265 (26%), 257 (24%), 253 (M-CH_2O-H_2O-H_2O-side chain; 26%); high resolution MS on ion at *m/e* 414, 414.3487 (calc. for $C_{28}H_{46}O_2$: 414.3497); MS of *tris*-TMS ether derivative, 648 (M; 10%), 633 (M-CH_3; 3%), 558 (M-trimethylsilanol; 72%), 554 (M-$CH_2OSi(CH_3)_3$; 7%), 543 (M-CH_3-trimethylsilanol; 27%), 468 (M-trimethylsilanol-trimethylsilanol; 60%), 455 (M-trimethylsilanol-$CH_2OSi(CH_3)_3$; 16%), 453 (M-CH_3-trimethylsilanol-trimethylsilanol; 15%), 445 (30%), 405 (21%), 378 (28%), 365 (M-trimethylsilanol-trimethylsilanol-$CH_2OSi(CH_3)_3$; 24%), 363 (30%), 355 (M-trimethylsilanol-trimethylsilanol-side chain; 17%), 343 (M-$CH_3OSi(CH_3)_3$-side chain; 8%), 281 (100%), 265 (19%), 248 (25%), high resolution MS, 648.4780 (calc. for $C_{37}H_{72}O_3{}^{28}Si_3$: 648.4789). The free sterol showed a single component on TLC in two solvent systems (50% benzene in ethyl acetate and 35% ethyl acetate in $CHCl_3$). The *tris*-TMS ether derivative showed a single component on GLC (3% OV-1 and OV-17; 270°C).

The contents of fractions 117 through 141 were pooled and evaporated to dryness at 35° under reduced pressure to give a white crystalline solid which was washed with hexane to give XIX (71 mg; 21% yield) melting at 225.5–227.0°C (after recrystallization from CH_2Cl_2 the compound melted at 227.0–228.5°); IR, ν_{max} 3380, 1670, 1077, 1051, 913, and 630 cm^{-1}; NMR, 0.77 (s, 3H, C-18-CH_3), 0.85 (s, 3H, C-19-CH_3), 3.17 (m, ~0.7H, H-bonded hydroxyl proton; disappeared upon exchange with D_2O); 3.58 (m, ~1.2H, C-3-H and C-32-H; integrated for 1H upon exchange with D_2O); 3.93 (m, ~1.8H, C-32-H_2; integrated for 2H upon exchange with D_2O), 4.33 (m, 1H, C-15-H), and 5.78 (m, 1H, C-7-H); MS, 414 (M-H_2O; 6%), 401 (M-CH_2OH; 15%), 399 (M-CH_3-H_2O; 5%), 396 (M-H_2O-H_2O; 5%), 384 (M-CH_2O-H_2O; 100%), 383 (M-H_2O-CH_2OH; 67%), 369 (27%), 366 (M-CH_2O-H_2O-H_2O; 7%), 365 (9%), 301 (M-H_2O-side chain; 5%), 271 (M-CH_2O-H_2O-side chain; 74%), 270 (M-H_2O-CH_2OH-side chain; 9%), 257 (29%), 253 (M-CH_2O-H_2O-H_2O-side chain; 16%); high resolution MS on ion at *m/e* 414, 414.3503 (calc. for $C_{28}H_{46}O_2$: 414.3500); MS of *tris*-TMS ether derivative 633 (M-CH_3; 35%), 558 (M-trimethylsilanol; 14%), 545 (M-$CH_2OSi(CH_3)_3$; 8%), 543 (M-CH_3-trimethylsilanol; 10%), 468 (M-trimethylsilanol-trimethylsilanol, 34%), 455 (M-trimethylsilanol-$CH_2OSi(CH_3)_3$; 81%), 453 (M-CH_3-trimethylsilanol-trimethylsilanol; 12%), 445 (18%), 391 (100%), 365 (M - trimethylsilanol - trimethylsilanol - CH_2OSi - $(CH_3)_3$; 46%), 355 (M-trimethylsilanol-trimethylsilanol-side chain; 20%), 315 (25%), 281 (43%), and 265 (38%); high resolution MS on ion at *m/e* 633: 633.4549 (calc. for $C_{37}H_{72}O_3{}^{28}Si_3$: 633.4554). The free sterol showed a single component on TLC in two solvent systems (50% benzene in ethyl acetate and 35% ethyl acetate in $CHCl_3$). The *tris*-TMS ether derivative showed a purity of ~99% on GLC (3% OV-1 and 3% OV-17, 270°C).

The contents of fractions 245 through 285 were pooled and evaporated to dryness at 35°C under reduced pressure to give a white crystalline solid which was washed with hexane to give XX (80 mg; 24% yield) which melted at 213–214°C; IR, ν_{max} 3430, 1051, 880, and 838 cm^{-1}; NMR 0.75 (s, 3H, C-18-CH_3), 0.88 (s, 3H, C-19-CH_3), 3.56 (m, 1H, C-3-H), and 4.07 (m, 4H, C-32-H_2, C-7β-H, and C-15-H); MS, 432 (M; 2%), 414 (M-H_2O; 58%), 401 (M-CH_2OH; 18%), 399 (M-CH_3-H_2O; 23%), 396 (M-H_2O-H_2O; 7%), 386 (M-CH_3-CH_2OH; 57%), 384 (M-CH_2O-H_2O; 100%), 381 (M-CH_3-H_2O-H_2O; 8%), 371 (M-CH_3-CH_3-CH_2OH; 28%), 369 (M-CH_3-CH_2O-H_2O; 18%), 365 (10%), 329 (10%), 319 (M-side chain; 2%), 301 (M-H_2O-side chain; 54%), 273 (M-H_2O-H_2O-side chain; 60%), 271 (M-CH_2O-H_2O-side chain; 55%), 263 (19%), 255 (20%), 253 (M-CH_2O-H_2O-H_2O-side chain; 12%); high resolution MS, 432.3580 (calc. for $C_{28}H_{48}O_3$: 432.3572); MS of *bis*-TMS ether derivative, 576 (M; 6%), 561 (M-CH_3; 24%), 545 (M-CH_2OH; 4%) 486 (M-trimethylsilanol; 100%), 471 (M-CH_3-trimethylsilanol; 14%), 458 (34%), 456 (M-CH_2O-trimethylsilanol; 36%), 455 (M-CH_2OH-trimethylsilanol; 15%), 396 (M-trimethylsilanol-trimethylsilanol; 9%), 373 (M-trimethylsilanol-side chain; 16%), 365 (M-CH_2OH-trimethylsilanol-trimethylsilanol; 10%), 345 (92%), 333 (57%), 283 (M-trimethylsilanol-trimethylsilanol-side chain; 11%), 255 (56%), 243 (50%), 241 (75%), 225 (29%), and 213 (39%). The compound showed a single component on TLC in two solvent systems (ethyl acetate and 50% benzene in ethyl acetate). The TMS ether derivative showed a purity of ~98.5% on GLC (3% OV-1 and 3% OV-17). The free sterol had the same chromatographic mobility on TLC (solvent systems, 50% benzene in ethyl acetate and ethyl acetate) as a sample of XX obtained directly by $LiAlH_4$ reduction of XVII. The *bis*-TMS ether derivatives of the two samples showed the identical chromatographic behavior on GLC (3% OV-1 and 3% OV-17; 270°C) and the same mass spectral properties on GLC–MS analyses.

14α,Hydroxymethyl-5α-cholest-6-en-3-one (IX). To component 1 (100 ml) of the Bio-Dynamics (BMC

Division) Cholesterol Auto Test was added cholesterol oxidase (3.2 ml; component 3 of the Cholesterol Auto Test) and the resulting mixture was diluted with water (100 ml). Compound VII (29.7 mg; 0.071 mmol) in isopropanol (10 ml) was added and the resulting mixture was incubated for 5.5 hr with shaking at 37°C. The mixture was extracted four times with ether (100 ml portions) and the combined extracts were dried over $MgSO_4$. Evaporation of the solvent gave a yellow oil which was subjected to silica gel (60–200 mesh; 40 g; packed as a slurry in benzene) column (45 cm × 1.3 cm) chromatography using 30% ether in benzene to apply the sample to the column and as the eluting solvent. Fractions 10 ml in volume were collected. The contents of fractions 8 through 15 were pooled and, after evaporation of the solvent, subjected to silica gel (40 g; packed as a slurry in hexane) column (45 cm × 1.3 cm) chromatography using 50% ether in hexane to apply the sample to the column and as the eluting solvent. Fractions 19 ml in volume were collected. The contents of fractions 11 and 12 were rechromatographed on a similar column. All of the fractions containing pure product (as judged by TLC) were pooled and after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give IX (23.5 mg; 80% yield) melting at 164–165°C; IR, ν_{max} 3440, 2955, 1710 (ketone in 6-membered ring), 1470, and 1039 cm^{-1}; MS, 414 (M; 13%), 396 (M-H_2O; 36%), 383 (M-CH_2OH; 100%), 365 (M-H_2O-CH_2OH; 10%), 283 (M-H_2O-side chain; 15%), 271 (13%), 269 (16%), 259 (13%), 257 (11%), 243 (11%), 229 (24%), 228 (19%), 217 (10%), and 207 (28%); high resolution MS, 414.3495 (calc. for $C_{28}H_{46}O_2$: 414.3498). The compound showed a single component on TLC (solvent, 50% ether in benzene; R_f 0.42). Analysis by GLC (3% OV-17; 300°C) indicated the purity to be ~97%.

14α-Hydroxymethyl-5α-cholest-7-en-3-one (X). To component 1 (300 ml) of the Biodynamics (BMC Division) Cholesterol Auto Test was added cholesterol oxidase (10 ml; component 3 of the Cholesterol Auto Test) and the resulting mixture was diluted with water (290 ml). Compound VII (122 mg; 0.293 mmol) in isopropanol (30 ml) was added, and the resulting mixture was incubated for 8.5 hr at 37°C with shaking. The crude product was isolated as described above and subjected to chromatography on a silica gel (60–200 mesh; packed as a slurry in 30% ether in benzene) column (45 cm × 2.0 cm) using the same solvent to apply the sample to the column and as the elution solvent. Fractions 20 ml in volume were collected. The contents of fractions 9 through 15 were pooled and rechromatographed on another silica gel (packed as a slurry in a mixture of ether and hexane (6:4)) column (45 cm × 1.3 cm) using the same solvent mixture for sample application and elution. Fractions 11 ml in volume were collected. The contents of fractions 24 through 75 were pooled and, after evaporation of the solvent, recrystallized from acetone to give *X* (94.7 mg; 78% yield) melting at 175.5–176.5°; IR, ν_{max} 3550, 2960, 1720 (ketone in 6-membered ring), 1468, 1383, and 1030 cm^{-1}; NMR, 0.75 (s, 3H, C-18-CH_3), 1.05 (s, 3H, C-19-CH_3), 2.23 (m, 4H, C-2-H_2 and C-4-H_2), 3.24 (dd, 1H, C-32-H; J = 10 c.p.s., J = 10 c.p.s.; collapsed to d, 1H, J = 10 c.p.s. upon exchange with D_2O), 3.66 (d, 1H, C-32-H; J = 10 c.p.s.), and 5.32 (m, 1H, C-7-H); MS, 414 (M; 3%), 399 (M-CH_3; 2%), 396 (M-H_2O; 2%), 383 (M-CH_2OH; 100%), 369 (M-CH_2O-CH_3; 11%) 365 (M-CH_2OH-H_2O; 6%), 273 (10%), 271 (21%), 257 (27%), 243 (20%), 231 (11%), 229 (26%), and 217 (24%); high resolution MS on ion at *m/e* 383, 383.3313 (calc. for $C_{27}H_{43}O$: 383.3314). The compound showed a single component on TLC (solvent, 50% ether in benzene; R_f 0.27). Analysis by GLC (3% OV-17, 300°C) indicated the purity to be in excess of 98%.

14α-Hydroxymethyl-5α-cholest-7-en-15α-ol-3-one (XXI). To component 1 (150 ml) of the Biodynamics (BMC Division) Cholesterol Auto Test was added cholesterol oxidase (4.8 ml; component 3 of the Cholesterol Auto Test) and the resulting mixture was diluted with water (150 ml). Compound XIX (51.6 mg; 0.12 mmol) in isopropanol (15 ml) was added and the resulting mixture was incubated, with gentle stirring, for 22 hr at room temperature. Water (200 ml) was added and the resulting mixture was extracted four times with $CHCl_3$ (100 ml portions). The combined organic extracts were dried over $MgSO_4$. Evaporation of the solvent gave an orange oil which was subjected to chromatography on an activated silicic acid (25 g; Unisil; Clarkson Chemical Company) column. The column (1.1 cm × 36 cm) was packed as a slurry in benzene which was also used for sample application. Using a mixture of ether and benzene (1:1) as the eluting solvent, fractions ~13 ml in volume were collected.The contents of fractions 8 through 19 were pooled and, after evaporation of the solvent under reduced pressure, recrystallized from acetone-water to give XXI (37.6 mg; 73% yield) as fine needles melting at 152.5–153.5°C, IR, ν_{max}, 3410, 2945, 1713 (ketone in 6-membered ring), 1443, 1386, and 1072 cm^{-1}; NMR, 0.79 (s, 3H, C-18-CH_3) 1.05 (s, 3H, C-19-CH_3), 2.24 (m, 4H, C-2-H_2 and C/4-H_2), 3.15 (m, ~0.7H, H-bonded hydroxyl proton disappeared on exchange with D_2O), 3.58 (m, ~1.2H, C-3-H and C-32-H), 3.93 (m, ~1.8H, C-32-H_2), 4.35 (m, 1H, C-15-H), and 5.78 (m, 1H, C-7-H); MS, 412 (M-H_2O; 1%), 399 (M-CH_2OH; 5%), 382 (M-H_2O-CH_2O; 85%), 381 (M-

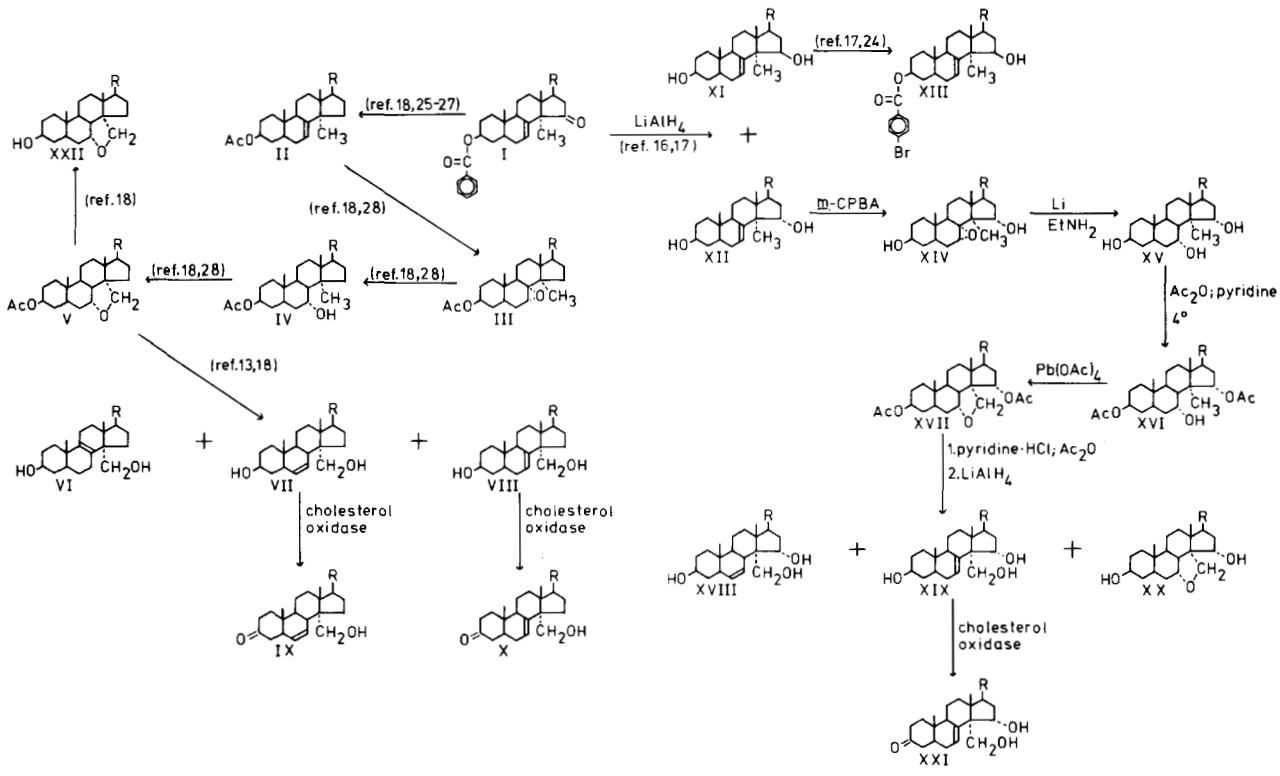


Fig. 1. Syntheses and stereochemical interrelationships of 14α-hydroxymethylsterols and 14α-hydroxymethyl-15α-hydroxysterols.

H_2O-CH_2OH; 20%), 367 (M-CH_2O-H_2O-CH_3; 19%), 269 (M-side chain-H_2O-CH_2O; 100%), 255 (32%), and 203 (11%); high resolution MS, 412.3345 (calc. for $C_{28}H_{44}O_2$: 412.3341). The compound showed a single component on TLC (solvent, ether).

RESULTS

Chemical Synthesis

The syntheses and stereochemical interrelationships of the various 14α-hydroxymethyl-substituted sterols and related compounds are outlined in **Fig. 1.** The syntheses of 14α-hydroxymethyl-5α-cholest-8-en-3β-ol (VI), 14α-hydroxymethyl-5α-cholest-6-en-3β-ol (VII), 14α-hydroxymethyl-5α-cholest-7-en-3β-ol (VIII), and 7α,32-epoxy-14α-methyl-5α-cholestan-3β-ol (XXII) have been described previously (10, 13, 18).[5] The preparation of the 14α-hydroxymethyl-Δ^6-3-ketosterol (IX) and of the 14α-hydroxymethyl-Δ^7-3-ketosterol (X) was effected in high yield by incubation of the corresponding 3β-hydroxysterol with cholesterol oxidase, an enzyme which we have previously found to be very useful on a preparative scale for the selective oxidation of the 3β-hydroxyl function of a number of 3β,15-dihydroxy and 3β,14α-dihydroxysterols (29–32). The syntheses of 14α-hydroxymethylsterols with 15α-hydroxyl functions utilized the same basic approach as in the case of the previously noted 14α-hydroxymethylsterols. Treatment of 14α-methyl-5α-cholest-7-en-3β,15α-diol (XII), obtained by hydride reduction of 3β-benzoyloxy-14α-methyl-5α-cholest-7-en-15-one (I), with *m*-chloroperbenzoic acid gave, in 93% yield, the desired 7α,8α-epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XIV). Reduction of XIV with lithium in ethylamine gave two products, 14α-methyl-5α-cholest-7-en-3β,15α-diol (XII; 37% yield) and the desired 14α-methyl-5α-cholestan-3β,7α,15α-triol (XV; 53% yield). Compound XII was identical in its physical and chromatographic properties to an authentic sample prepared by an alternative route. Compound XV, previously undescribed, was characterized by the results of spectral analyses. Moreover, selective acetylation of the 3β- and 15α-hydroxyl functions of XV gave 3β,15α-diacetoxy-14α-methyl-5α-cholestan-7α-ol (XVI) in high yield (84%) by treatment with acetic anhydride and pyridine at 4°C. Compound XVI was characterized by the results of standard spectral analyses and the presence of one free hydroxyl function in XVI was further confirmed by the results of mass spectral analyses of the TMS

[5] The syntheses of VII and VIII were also presented at three symposiums (19–21).

TABLE 1. C-18 and C-19 methyl group resonances for 14α-hydroxymethyl sterols and related sterols

Compound	C-18		C-19	
	δ_{obs}	δ_{calc}	δ_{obs}	δ_{calc}
14α-Hydroxymethyl-5α-cholest-7-en-3β-ol (VIII) (ref. 18)	0.70		0.835	
14α-Hydroxymethyl-5α-cholest-7-en-3-one (X)	0.75	0.76	1.05	1.04
14α-Hydroxymethyl-5α-cholest-8-en-3β-ol (VI) (ref. 18)	0.70	0.76	0.98	0.97
14α-Hydroxymethyl-5α-cholest-6-en-3β-ol (VII) (ref. 18)	0.92	0.90	0.82	0.82
14α-Hydroxymethyl-5α-cholest-7-en-3β,15α-diol (XIX)	0.77	0.76	0.85	0.84
14α-Hydroxymethyl-5α-cholest-7-en-15α-ol-3-one (XXI)	0.79	0.80	1.05	1.06
14α-Hydroxymethyl-5α-cholest-6-en-3β,15α-diol (XVIII)	0.95	0.95	0.81	0.83
7α,32-Epoxy-14α-methyl-5α-cholestan-3β-ol (XXII)	0.74		0.88	
7α,32-Epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XX)	0.75	0.77	0.88	0.89
3β,15α-Diacetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (XV)	0.82	0.82	0.88	0.88
14α-Methyl-5α-cholest-7-en-3β,15α-diol (XII)	0.70	0.68	0.81	0.81
14α-Methyl-5α-cholestan-3β,7α,15α-triol (XVII)	0.81	0.78	0.88	0.88
3β,15α-Diacetoxy-14α-methyl-5α-cholestan-7α-ol (XVI)	0.81	0.82	0.88	0.89
7α,8α-Epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XIV)	0.88	0.87	0.85	0.84

derivative of XVI. Compound XVI was converted, in 64% yield, to the desired 3β,15α-diacetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (XVII) by (XVII) by treatment with lead tetraacetate, a reaction first introduced by Fried, Brown, and Borkenhagen (34) for the formation of the desired tetrahydrofuran system from a 7α-hydroxy-14α-methylsterol. Fried et al. (34) also first demonstrated the acetolytic cleavage of the tetrahydrofuran system to give the acetylated derivative of the 14α-hydroxymethyl-Δ^7-sterol. In the present case treatment of the 7α,32-epoxide XVII with pyridine hydrochloride in refluxing acetic anhydride, followed by lithium aluminum hydride reduction of the resulting crude product, gave three products: 14α-hydroxymethyl-5α-cholest-6-en-3β,15α-diol (XVIII; 26% yield), 14α-hydroxymethyl-5α-cholest-7-en-3β,15α-diol (XIX; 21% yield), and 7α,32-epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XX; 24% yield). These compounds were fully characterized by spectral analyses and by GLC–MS analyses of the corresponding trimethylsilyl derivatives. The location of the nuclear double bond in XVIII at the Δ^6-position was indicated by the presence, in its NMR spectrum, of two olefinic proton resonances which formed an AX spin system (J_{AX} = 12 c.p.s.). The location of the nuclear double bond in XIX was indicated by the single olefinic proton resonance in its NMR spectrum. These assignments were further supported by the positions of the C-18 and C-19 methyl resonances in the NMR spectra of these compounds (vide infra). 14α-Hydroxymethyl-5α-cholest-7-en-15α-ol-3-one (XXI) was prepared in 80% yield from XIX upon incubation with cholesterol oxidase.

It is important to note that the configuration of the 14α-methyl group in I has been unequivocally established by X-ray crystallographic analysis of the *p*-bromobenzoate derivative (XIII) of one (XI) of the epimeric diols obtained upon lithium aluminum hydride reduction of I (17, 24). Since none of the subsequent reactions involved in the formation of the various 14α-hydroxymethylsterols from I involve cleavage of the carbon-carbon bond between C-14 and C-32 or inversion of the C-D ring junction, the configuration of the hydroxymethyl groups in the various sterols is as specified in each case. It is also important to note that the hydroxyl function at carbon atom 15 in XII has also been unambiguously established by the results of the X-ray crystallographic analysis of the 3β-*p*-bromobenzoate derivative of 14α-methyl-5α-cholest-7-en-3β,15β-diol (17, 24). Since none of the reactions involved in the formation of the various new 15α-hydroxysterols or their derivatives, i.e., 7α,8α-epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XIV), 14α-methyl-5α-cholestan-3β,7α,15α-triol (XV), 3β,15α-diacetoxy-14α-methyl-5α-cholestan-7α-ol (XVI), 3β,15α-diacetoxy-7α,32-epoxy-14α-methyl-5α-cholestane (XVII), 7α,32-epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XX), 14α-hydroxymethyl-5α-cholest-6-en-3β,15α-diol (XVIII), 14α-hydroxymethyl-5α-cholest-7-en-15α-ol-3-one (XXI), and 14α-hydroxymethyl-5α-cholest-7-en-3β,15α-diol (XIX), involve cleavage of the oxygen-carbon bond at C-15, the configuration of the hydroxyl functions in the various sterols is as specified in each case.

Further evidence in support of the nuclear double bond assignments and other structural assignments was derived from considerations of the positions of the C-18 and C-19 methyl resonances in the NMR spectra of these compounds (**Table 1**). Comparison of the NMR spectrum of VIII and its corresponding 3-ketosterol (X) in the light of Zurcher's tables (35) allowed unambiguous assignment of the angular methyl group resonances (18). Then, by using Zurcher's tables and the angular methyl groups of VIII

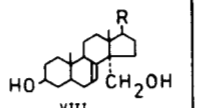
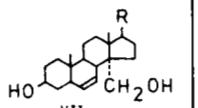
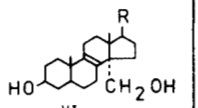
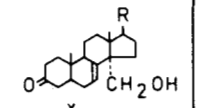
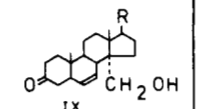
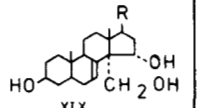
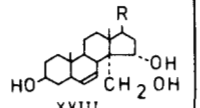
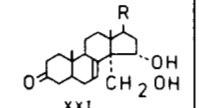
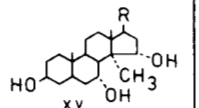
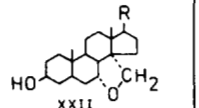
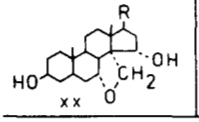
Inhibitor	Concentrations (μM) Required for 50% Inhibition			
	L Cell Cultures		Primary Cultures of Liver Cells	
	Sterol Synthesis	HMG-CoA Reductase	Sterol Synthesis	HMG-CoA Reductase
 VIII	2.0	3.3	10.0	8.0
 VII	0.2	0.5	3.0	2.0
 VI	4.0	6.8	>47	8.0
 X	4.0	2.0	0.7	4.0
 IX	0.8	1.0	0.7	2.0
 XIX	0.2	0.4	0.1	0.3
 XVIII	0.7	0.5	0.3	0.1
 XXI	0.8	1.0	—	—
 XV	0.7	2.0	6.0	2.0
 XXII	0.8	3.0	10.0	8.0
 XX	9	>74	7	>46

Fig. 2. Inhibition of sterol synthesis and reduction of HMG-CoA reductase activity by 14α-hydroxymethylsterols and by 14α-hydroxymethyl-15α-hydroxysterols. The values of the inhibitory potencies of these sterols with respect to sterol synthesis and HMG-CoA reductase were based upon analyses of plots of activity versus concentration of the sterol over a range of a minimum of four concentrations of the inhibitory sterol (see text and Figs. 3 and 4).

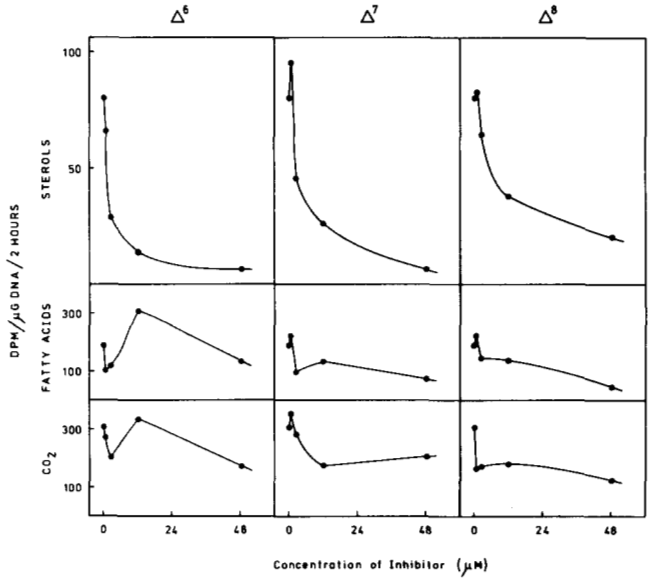


Fig. 3. Effects of 14α-hydroxymethyl-5α-cholest-6-en-3β-ol, 14α-hydroxymethyl-5α-cholest-7-en-3β-ol, and 14α-hydroxymethyl-5α-cholest-8-en-3β-ol on the incorporation of labeled acetate into digitonin-precipitable sterols, fatty acids, and carbon dioxide in primary cultures of fetal mouse liver cells.

as a base, it was possible to calculate the expected C-18 and C-19 methyl group resonances for the remaining sterols under consideration. The data presented in Table 1 show that the calculated and observed values for these resonances were in close agreement, thus supporting the structural assignments.

Cell culture studies

Inhibition of sterol synthesis and reduction of level of HMG-CoA reductase activity in L cells and in primary cultures of mouse liver cells by 14α-hydroxymethyl sterols. A number of 14α-hydroxymethyl sterols were found to be potent inhibitors of sterol biosynthesis in L cells and in primary cultures of fetal mouse liver cells (**Fig. 2**). **Fig. 3** illustrates the effects of 14α-hydroxymethyl-5α-cholest-6-en-3β-ol, 14α-hydroxymethyl-5α-cholest-7-en-3β-ol, and 14α-hydroxymethyl-5α-cholest-8-en-3β-ol on the rates of formation of CO_2, fatty acids, and digitonin-precipitable sterols in primary cultures of fetal mouse liver cells. The results show that the suppression of all three sterols on sterol biosynthesis was a function of their concentration in the medium. In contrast the rates of fatty acid synthesis and CO_2 production showed considerable variation with increasing concentration of

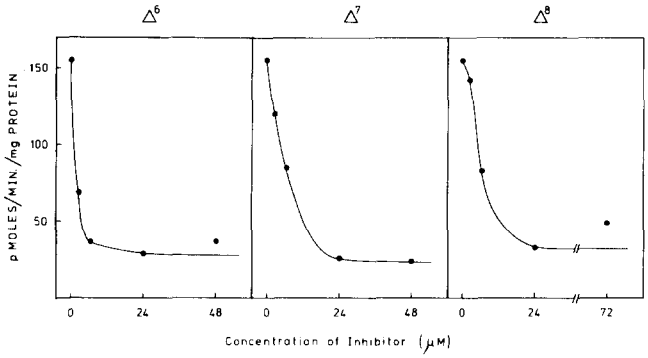


Fig. 4. Effects of 14α-hydroxymethyl-5α-cholest-6-en-3β-ol, 14α-hydroxymethyl-5α-cholest-7-en-3β-ol, and 14α-hydroxymethyl-5α-cholest-8-en-3β-ol on HMG-CoA reductase activity in primary cultures of fetal mouse liver cells.

the sterols. A more or less continuous reduction in the synthesis of fatty acids with increasing concentration of the Δ^8-sterol was observed which accompanied the moderate decrease in sterol synthesis with increasing concentrations of the same sterol. As a consequence (see Fig. 2) the ratio of sterol synthesis to fatty acid synthesis plotted as a function of the concentration of the Δ^8-sterol declined but did not drop as far as 50% of the control level. However, as noted in **Fig. 4,** the Δ^8-14α-hydroxymethyl sterol, as well as the corresponding Δ^7- and Δ^6-isomers, was qutie potent in causing a suppression of the levels of HMG-CoA reductase in the fetal liver cells. The combined data indicate that 14α-hydroxymethyl-5α-cholest-8-en-3β-ol is a potent inhibitor of sterol biosynthesis. Moreover, this sterol appears to have an additional inhibitory effect either on a reaction common to both sterol and fatty acid synthesis or on a reaction unique to fatty acid synthesis. The curves presented in Fig. 3 and the 50% inhibition values listed in Fig. 2 indicate that the Δ^6-sterol was a more potent inhibitor of sterol synthesis in the liver cell cultures than the corresponding Δ^7 and Δ^8 sterols. The data in Fig. 2 also show that the Δ^6-diol was the most potent of the three inhibitors in the L cell cultures. Oxidation of the 3β-hydroxyl function of the Δ^7-diol and of the Δ^6-diol to give the corresponding 3-ketosterols 14α-hydroxymethyl-5α-cholest-7-en-3-one (X) and 14α-hydroxymethyl-5α-cholest-6-en-3-one (IX) did not markedly affect the ability to suppress HMG-CoA reductase activity as compared with the corresponding diols but it did result in an increase in ability to inhibit cholesterol synthesis in the liver cell cultures. Introduction of a 15α-hydroxyl function to give the 3β,15α,32-triol increased inhibitory activity over that found with the corresponding diol when the double bond was in the Δ^7-position (XIX) but not when it was in the Δ^6-position (XVIII). Comparison of the results obtained with the 3β,15α,-32-triols with those for the saturated 14α-methyl-3β,7α,15α-triol (XV) indicated that although the inhibitory activities observed may have been influenced quantitatively by the presence of a 14α-hydroxymethyl group and by the position of a nuclear double bond, they were not entirely determined by these structural features. It is worthy of note that although 7α,32-epoxy-14α-methyl-5α-cholestan-3β-ol (XXII) suppressed HMG-CoA reductase activity in both cell types, the corresponding 15α-hydroxy compound (XX) inhibited sterol synthesis at some site other than the reductase.

DISCUSSION

We have previously reported that a large number of 15-oxygenated sterols are potent inhibitors of sterol biosynthesis in L cells and in primary cultures of fetal mouse liver cells (22, 29, 30, 33, 36–38). The inhibition of sterol synthesis by these compounds was associated with a reduction in the levels of HMG-CoA reductase activity. The results presented herein establish that 15α-hydroxy-Δ^7-sterols with a 14α-hydroxymethyl group (compounds XIX and XXI) were also potent inhibitors of sterol synthesis in L cells and in primary cultures of fetal mouse liver cells and caused a reduction of the levels of HMG-CoA reductase activity in the same cells (Fig. 2). The inhibition of sterol synthesis and the reduction of the levels of HMG-CoA reductase by 14α-methyl-5α-cholestan-3β,7α,15α-triol is worthy of note. This finding constitutes the first demonstration of such effects by a 14α-alkyl substituted 15-oxygenated sterol which lacks a double bond in the sterol nucleus. Very recently we have also observed the inhibition of sterol synthesis in L cells by C_{27} 15-oxygenated sterols which contained no olefinic double bonds in the sterol nucleus (33). The effects of one 15α-hydroxysterol were unusual. 7α,32-Epoxy-14α-methyl-5α-cholestan-3β,15α-diol (XX) was moderately active in the inhibition of sterol synthesis in L cells and in primary cultures of fetal mouse liver cells. However, the compound had little or no effect on the levels of HMG-CoA reductase in the same cells. This combination of findings is unique among all of the 15-oxygenated sterols studied to date and indicates an inhibition of sterol synthesis at a site or sites not involving the important regulatory enzyme HMG-CoA reductase.

Although the precise mechanisms involved in the removal of the 14α-methyl group of cholesterol precursors have not been conclusively established, the sequence of events that results in the enzymatic re-

moval of this methyl group appears to involve an initial hydroxylation to yield the 14α-hydroxymethyl sterol, subsequent formation of the corresponding aldehyde, followed by elimination of carbon atom 32 as formic acid to yield $\Delta^{8,14}$ or $\Delta^{7,14}$ sterols and/or $\Delta^{8(14)}$ sterols (7, 9–12, 16, 17, 19–21, 27, 39–65). The possible involvement of 15-hydroxy derivatives in the overall enzymatic removal of carbon atom 32 of cholesterol precursors has also been considered by several laboratories (7, 10–12, 16, 17, 19–21, 24, 48, 53–57, 59). The precise stage at which 15-hydroxylation occurs, if it indeed does occur, is not known. Thus, while considerable uncertainty exists with respect to the detailed mechanisms involved in the overall enzymatic removal of the 14α-methyl group of cholesterol precursors, available evidence indicates that the initial enzymatic reaction at this carbon atom is a hydroxylation to yield the corresponding 14α-hydroxymethyl sterol. The results presented herein clearly demonstrate that 14α-hydroxymethyl sterols are potent inhibitors of the biosynthesis of digitonin-precipitable sterols and cause a reduction of the levels of HMG-CoA reductase activity in L cells and in primary cultures of fetal mouse liver cells. 14α-Hydroxymethyl-5α-cholest-7-en-3β-ol has been shown to be very efficiently converted to cholesterol upon incubation with rat liver homogenate preparations (10, 19–21).

While we have not investigated the metabolism of 14α-hydroxymethyl-5α-cholest-8-en-3β-ol in a similar fashion we consider it extremely probable, in view of the results of studies of the metabolism of 5α-lanost-8-en-3β,32-diol (11, 12), that the same situation exists as in the case of the metabolism of the corresponding Δ^8-isomer. We have previously shown that while Δ^8- and Δ^7-cholestenols serve as efficient precursors of cholesterol upon incubation with rat liver homogenates (66, 67), the corresponding Δ^6 sterol is not convertible to cholesterol upon intraportal injection or upon feeding to intact rats (67). The high activity of 14α-hydroxymethyl-5α-cholest-6-en-3β-ol in the inhibition of sterol biosynthesis relative to the corresponding Δ^8 and Δ^7 isomers may be explainable in terms of a more rapid metabolism of the latter sterols. In view of the possible rapid metabolism of the Δ^8- and Δ^7-hydroxymethyl sterols to cholesterol in the cell culture systems studied, the actual intracellular concentrations of these sterols that are required to suppress reductase activity and inhibit sterol biosynthesis may be very much lower than those shown in Fig. 1 and Table 1. It is worthy of note that addition of a 15α-hydroxyl function to 14α-methyl-5α-cholest-6-en-3β-ol had no significant effect on the inhibitory potency, while the addition of a 15α-hydroxyl function to 14α-hydroxymethyl-5α-cholest-7-en-3β-ol caused an increase in the inhibitory potency in both the L cells and in the primary cultures of fetal mouse liver cells (Table 1). The higher potency of the 15α-hydroxy derivative of 14α-hydroxymethyl-5α-cholest-7-en-3β-ol may be related to the more rapid metabolism of the 14α-hydroxymethyl-5α-cholest-7-en-3β-ol in the cells since we have recently observed that no conversion of the corresponding 3β,15α,32-triol to cholesterol could be detected upon incubation with homogenate preparations of rat liver.[6]

The fact that 14α-hydroxymethyl sterol precursors of cholesterol are potent inhibitors of sterol biosynthesis has important potential implications with regard to the regulation of cholesterol biosynthesis and effects derived from this process. As noted previously, oxygenated metabolites of cholesterol have been shown to be potent inhibitors of cholesterol biosynthesis and have been considered to be potential regulators of sterol biosynthesis in the cells in which they are formed from cholesterol (1–4). Moreover, the enzymatic formation of these oxygenated sterols from cholesterol may be organ specific, for example, 7-oxygenated sterols in liver and 20- or 22-oxygenated sterols in organs capable of steroid hormone synthesis. However, on the basis of our current knowledge of the biosynthesis of cholesterol, 14α-hydroxymethyl sterols should be formed in all cells which are capable of the de novo biosynthesis of cholesterol. These factors are important in considerations of the possible role of 14α-hydroxymethyl sterols in the regulation of the biosynthesis of cholesterol and in the regulation of other important processes requiring the synthesis of cholesterol. For example, a cycle of sterol synthesis has been observed in mitogen-stimulated lymphocytes (4, 68). This cycle of sterol synthesis, which precedes DNA synthesis and cell division, can be inhibited by oxygenated derivatives of cholesterol. Since lymphocytes (and most other cell types) are not known to oxygenate cholesterol to any of the established inhibitors of cholesterol synthesis, regulation of cholesterol synthesis and hence of cell replication in these cells may involve an oxygenated sterol precursor of cholesterol such as 14α-hydroxymethyl sterol precursors of cholesterol. The implication of this suggestion is that different oxygenated sterols may be involved in regulating sterol synthesis that is required for cell division, for bile acid production, and for steroid hormone production. The latter hypothesis is not inconsistent with present understanding of those processes.

[6] Pascal, R. A., Jr., and G. J. Schroepfer, Jr. Manuscript in preparation.

This work was supported in part by grants HL-15376, HL-22532, and RR-07103 from the National Institutes of Health and by grant CA-02758 from the National Cancer Institute.

Manuscript received 11 October 1979 and in revised form 25 January 1980.

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