Inhibition of sterol biosynthesis in animal cells by 14α-alkyl-substituted 15-oxygenated sterols

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Abstract Reported herein are the results of investigations of the effects of a number of 14α-alkyl-substituted 15-oxygenated sterols, prepared by chemical synthesis, on sterol biosynthesis and the levels of 3-hydroxy-3-methylglutaryl CoA reductase activity in L cells and in primary cultures of fetal mouse liver cells grown in serum-free media. Several of the compounds, most notably 14α-ethyl-5α-cholest-7-en-3β,15α-diol and 14α-ethyl-5α-cholest-7-en-15α-ol-3-one, were found to be extraordinarily potent inhibitors of sterol synthesis in these cells. For example, the latter compound caused a 50% inhibition of the incorporation of labeled acetate into digitonin-precipitable sterols in L cells in culture at a concentration of 6×10^{-9} M. **—Schroepfer, G. J., Jr., E. J. Parish, M. Tsuda, D. L. Raulston, and A. A. Kandutsch.** Inhibition of sterol biosynthesis in animal cells by 14α-alkyl-substituted 15-oxygenated sterols. *J. Lipid Res.* 1979. **20:** 994–998.

Supplementary key words mouse L cells · fetal mouse liver cells · HMG-CoA reductase

A number of oxygenated derivatives of cholesterol and related sterols have been shown to act as potent inhibitors of sterol biosynthesis in animal cells in culture (1–12). Recently we have found that a number of 15-oxygenated sterols serve as very potent inhibitors of sterol synthesis in animal cells (8–12). One of the more potent inhibitors of this type, identified in initial studies, was 14α-methyl-5α-cholest-7-en-3β,15α-diol which caused a 50% inhibition of sterol synthesis in L cells and in primary cultures of liver cells at 3.0×10^{-7} M and 1.8×10^{-6} M, respectively (9). The same sterol resulted in a 50% reduction of the activity of HMG-CoA reductase in the L cells and in the mouse liver cells at concentrations of 3.0×10^{-7} M and 2.0×10^{-6} M, respectively (9).

As an extension of our initial studies of 15-oxygenated sterols as inhibitors of sterol synthesis, we have investigated the effects of additional 14α-alkyl substituted 15-oxygenated sterols and their derivatives on sterol synthesis in L cells and fetal mouse liver cells grown in serum-free media. A number of the new compounds studied have been found to be extraordinarily active in the inhibition of sterol synthesis. Preliminary accounts of portions of this research have been published (10, 11).

EXPERIMENTAL PROCEDURE

Materials

A preliminary account of the chemical syntheses of 14α-ethyl-5α-cholest-7-en-3β-ol-15-one, 14α-ethyl-5α-cholest-7-en-3β,15α-diol, and 14α-ethyl-5α-cholest-7-en-3β,15β-diol has been presented (10). The preparation of 14α-ethyl-5α-cholest-7-en-15α-ol-3-one from 14α-ethyl-5α-cholest-7-en-3β,15α-diol through the use of cholesterol oxidase has been fully described in a preliminary communication (11). A detailed description of the chemical syntheses and characterizations of all new compounds involved in the present study will be reported elsewhere (13).

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-cm² culture flasks as previously described (1, 2).

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, 9) except

Abbreviation: HMG, 3-hydroxy-3-methylglutaryl.

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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-labeled sterol recoveries. Studies of rates of acetate metabolism to fatty acids and, in some cases, to CO_2 were also made to detect any possible effects of the inhibitors of sterol synthesis on general metabolism. In the present series of tests, none of the compounds examined showed any 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-labeled sterols to ^{14}C-labeled fatty acids as a function of the concentration of the inhibitor (1). HMG-CoA reductase activity in the L cells and in the fetal mouse liver cells was assayed by a minor modification (14) of procedures described previously (1, 2, 9).

RESULTS

A number of the 14α-alkyl-substituted sterols were found to be very potent inhibitors of sterol synthesis in the L cells and in primary cultures of fetal mouse liver cells. The effects of one of these inhibitors, 14α-ethyl-5α-cholest-7-en-3β-ol-15-one, on the rates of [1-^{14}C]acetate metabolism to fatty acids and sterols, the ratios of synthesis of ^{14}C-labeled sterols to ^{14}C-labeled fatty acids, and HMG-CoA reductase activity in the L cells and in the primary cultures of mouse liver cells are shown in **Fig. 1.**

Table 1 presents a condensed summary of the results of tests of the inhibitory effects of twelve 14α-alkyl-Δ^7-unsaturated-3β-hydroxy-15-oxygenated sterols upon the rate of sterol synthesis from acetate and upon the level of HMG-CoA reductase activity in the two types of cell cultures. All of these 15-oxygenated sterols inhibited sterol synthesis and, with the exception of 14α-ethyl-5α-cholest-7-en-3β,15α-diol, the liver cells appeared to be more resistant to their effects than were the L cells. The most potent of these inhibitors of sterol synthesis was 14α-ethyl-5α-cholest-7-en-3β,15α-diol. The inhibitory effects of eight additional derivatives of this sterol on sterol synthesis and on HMG-CoA reductase activity in the L cells are summarized in **Table 2.** The extraordinarily high inhibitory activities of 14α-ethyl-5α-cholest-7-en-15α-ol-3-one and 3β-acetoxy-14α-ethyl-5α-cholest-7-en-15α-ol are especially noteworthy.

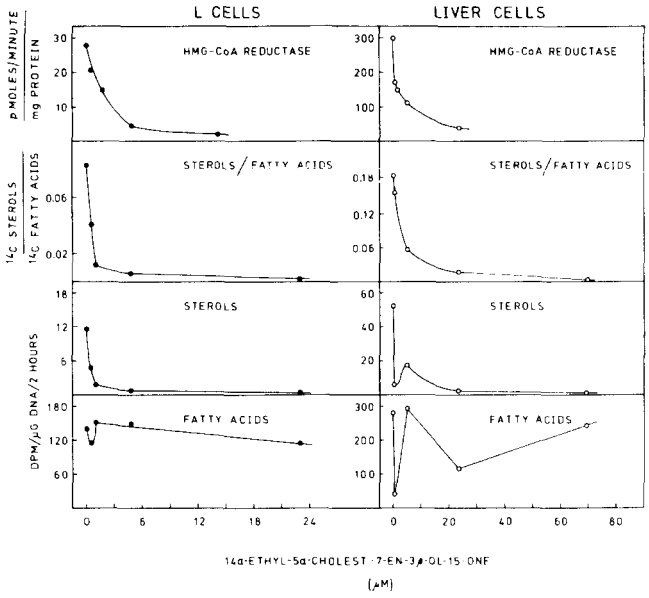
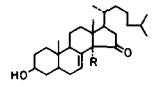
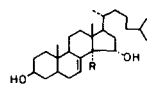
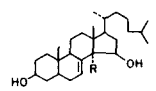


Fig. 1. Effects of 14α-ethyl-5α-cholest-7-en-3β-ol-15-one on (from bottom to top) the rate of [1-^{14}C]acetate metabolism to fatty acids and sterols, the ratio of ^{14}C-labeled sterols to ^{14}C-labeled fatty acids, and on HMG-CoA reductase activity in L cells (left) and in primary cultures of fetal mouse liver cells (right).

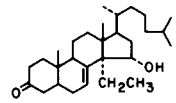
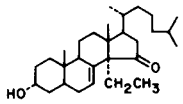
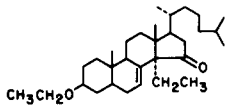
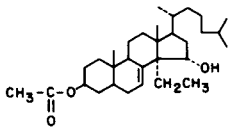
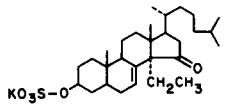
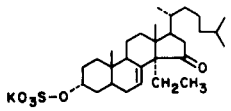
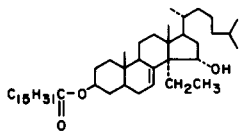
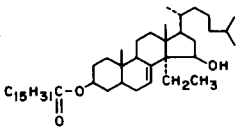
TABLE 1. Inhibition of sterol synthesis and reduction of HMG-CoA reductase activity in L cells and in primary cultures of fetal mouse liver cells by 14α-alkyl-substituted 15-oxygenated derivatives of 5α-cholest-7-en-3β-ol

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
	R=CH$_3$*	0.3	0.3	4.5	2.8
	R=CH$_2$CH$_3$	0.3	1.9	2.1	2.1
	R=CH$_2$CH$_2$CH$_3$	3.4	8.1	4.5	—
	R=CH$_2$CH$_2$CH$_2$CH$_3$	8.3	>13.0	11.0	—
	R=CH$_3$*	0.3	0.3	1.8	2.0
	R=CH$_2$CH$_3$	0.05	0.2	0.06	2.3
	R=CH$_2$CH$_2$CH$_3$	1.1	6.8	2.5	—
	R=CH$_2$CH$_2$CH$_2$CH$_3$	5.0	6.6	10.0	—
	R=CH$_3$*	0.5	1.2	—	4.3
	R=CH$_2$CH$_3$	0.4	3.5	0.8	7.9
	R=CH$_2$CH$_2$CH$_3$	4.1	8.1	12.0	—
	R=CH$_2$CH$_2$CH$_2$CH$_3$	4.4	13.0	15.0	—

* Data from Schroepfer et al. (9).

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 vs. concentration of the sterol over a range of a minimum of four concentrations of the inhibitory sterol (see text and Fig. 1).

TABLE 2. Inhibition of sterol synthesis and reduction of HMG-CoA reductase activity in L cells by derivatives of 14α-ethyl-5α-cholest-7-en-3β,15α-diol

Inhibitor	Concentrations (μM) Required for 50% Inhibition in L Cells	
	Sterol Synthesis	HMG-CoA Reductase
	0.006	0.05
	0.2	0.6
	>20.0	—
	0.03	0.13
	5.0	>10.0
	>11.0	—
	7.0	>15.0
	>15.0	—

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 vs. concentration of the sterol over a range of a minimum of four concentrations of the inhibitory sterol (see text and Fig. 1).

DISCUSSION

We have previously shown that 15-oxygenated derivatives of 14α-methyl-5α-cholest-7-en-3β-ol are potent inhibitors of sterol biosynthesis in animal cells in culture (8, 9). The 15α-hydroxy epimer appeared to be more active than the corresponding 15β-hydroxy epimer in the inhibition of sterol synthesis and in reduction of the level of HMG-CoA reductase activity (9). Since the 14α-methyl-5α-cholest-7-en-3β,15β-diol, but not 14α-methyl-5α-cholest-7-en-3β,15α-diol, was converted to cholesterol upon incubation with rat liver homogenate preparations (15, 16), we reasoned that the lower inhibitory potency of the 15β-hydroxy compound might be due to a slower rate of metabolism of the 15α-hydroxy compound in the test cells. As an extension of this line of reasoning we decided to explore the possible inhibitory action of a number of 15-oxygenated sterols having "unnatural" alkyl substitution in the 14α-position. As noted herein, many of these new 14α-alkyl-substituted 15-oxygenated sterols have been found to be very potent inhibitors of sterol synthesis in animal cells in culture.

The results summarized in Table 1 show that, with a few exceptions, the order of inhibitory potency for the 14α-alkyl-substituted 15-oxygenated sterols with respect to the synthesis of digitonin-precipitable sterols was ethyl > methyl > *n*-propyl > *n*-butyl. The 14α-alkyl-5α-cholest-7-en-3β,15α-diols were, in general, more potent than the corresponding 15β-hydroxy epimers with respect to their inhibitory action on sterol synthesis and on HMG-CoA reductase activity. As is shown in Table 2, the 15α-hydroxy derivative of 3β-hexadecanoyloxy-14α-ethyl-5α-cholest-7-ene was also more potent than the 15β-hydroxy epimer. Similar findings have also been observed in the cases of the 15-hydroxy derivatives of 3β-methoxy-14α-methyl-5α-cholest-7-ene (9).

Of the 14α-alkyl-Δ^7-3β,15-diols studied, the activities of 14α-ethyl-5α-cholest-7-en-3β,15α-diol were especially noteworthy. This compound was extremely potent as an inhibitor of acetate incorporation into digitonin-precipitable sterols in both the L cells and the fetal mouse liver cell cultures. In view of the high inhibitory potency of 14α-ethyl-5α-cholest-7-en-3β,15α-diol (Table 1) we explored the activities of a number of derivatives of this compound. The corresponding 3-keto derivative was extremely potent in the inhibition of sterol synthesis (Table 2). The 3β-acetoxy derivative of the 14α-ethyl-5α-cholest-7-en-3β,15α-diol had a similar inhibitory activity in the L cells as did the free sterol (Table 2) while the 3β-hexadecanoyloxy-derivative of the same compound was considerably less potent than the free sterol or the 3β-acetate derivative.

A number of derivatives of 14α-ethyl-5α-cholest-7-en-3β-ol-15-one were also studied. The corresponding 3α-hydroxy epimer had inhibitory activity comparable to that of the 3β-hydroxy compound. Inhibitory action of the 14α-ethyl-Δ^7-3β-ol-15-ketosterol was greatly diminished upon formation of the corresponding 3β-ethoxy derivative (Table 2), a finding similar to that made previously in the cases of the 14α-methyl-Δ^7-3β-hydroxy-15-ketosterol and its 3β-methyl ether (9). Formation of the sulfate ester resulted in a significant decrease in inhibitory activity relative to the free sterol which was even more marked in the case of the sulfate ester of the 3α-hydroxy compound. All of the sterols covered in this and our previous reports (8–12) on the inhibitory effects of 14α-alkyl substituted 15-oxygenated sterols on sterol biosynthesis in these cell culture systems have possessed a Δ^7 double bond. We have recently observed that a saturated 14α-alkyl-substituted 15-oxygenated sterol, 14α-methyl-5α-cholestan-3β,7α,15α-triol, is also a potent inhibitor of sterol synthesis in the L cells in culture.[5]

It is noteworthy that the concentrations of the sterols that were required for 50% inhibition of the incorporation of acetate into digitonin-precipitable sterols by the 14α-alkyl-substituted 15-oxygenated sterols were often lower than the concentrations required to cause a 50% reduction of the levels of HMG-CoA reductase activity in the cells, a finding suggesting that, in the cases of several of these 14α-alkyl-15-oxygenated sterols, the effects of these sterols were not limited to that upon HMG-CoA reductase activity. Relative to this point we have recently found that a number of the 14α-alkyl substituted 15-oxygenated sterols, when incubated with the 10,000 *g* supernatant fraction of rat liver homogenate preparations in the presence of appropriate cofactors, cause an inhibition of the incorporation of labeled mevalonate into digitonin-precipitable sterols.[6] This finding, suggesting the presence of a second site of action in the inhibition of sterol biosynthesis by these 14α-alkyl-15-oxygenated sterols, is currently being explored in depth.

We have previously reported that one 15-oxygenated sterol, 5α-cholest-8(14)-en-3β-ol-15-one, and several derivatives of this compound are effective in lowering serum cholesterol levels of intact animals upon oral or upon subcutaneous administration (17–20). The present report concerns a series of 14α-alkyl-substituted 15-oxygenated sterols, some of which, most notably 14α-ethyl-5α-cholest-7-en-3β,15α-diol and 14α-ethyl-5α-cholest-7-en-15α-ol-3-one, were found to be extraordinarily active in the inhibition of sterol synthesis in the cells in culture. We have recently observed that the former compound, upon subcutaneous administration, causes a significant reduction of the serum cholesterol levels of normal rats.[7] Apart from their obvious potential use in lowering serum cholesterol levels, the 15-oxygenated sterols with extraordinarily high potency in inhibition of sterol synthesis and in reduction of the levels of HMG-CoA reductase may find utility in the control of cellular replication. The inhibition of replication of L cells, fetal mouse liver cells, rat hepatoma cells, and phytohemagglutinin-stimulated mouse lymphocytes in culture by oxygenated derivatives of cholesterol has been reported previously (3, 4, 21). Thus, compounds with very high activity in the reduction of HMG-CoA reductase activity and in the inhibition of sterol synthesis, such as the 15-oxygenated compounds described herein, may, by preventing the formation of sterol required for membrane formation and the formation of essential products of mevalonate metabolism (i.e., isopentenyl residues of modified adenine residues in RNA, polyisoprenoid side chains of coenzyme Q, and other polyisoprenoid derivatives such as dolichol phosphate), prove to be useful in the control of normal and abnormal cell growth. The results of recent experiments indicate that 15-oxygenated sterols cause inhibition of the growth of L cells in culture.[8] Further studies of the metabolism, mechanisms of action, and effects of the 14α-alkyl-15-oxygenated sterols described herein are in progress.

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