Biological Evaluation *in Vivo* and *in Vitro* of Selected 5α-Cholestane-3β,5α,6β-triol Analogs as Hypocholesterolemic Agents[1a-c]

DONALD T. WITIAK,* ROGER A. PARKER,[1d-f] DOUGLAS R. BRANN,[1g]

Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210

MARY E. DEMPSEY, MARY C. RITTER,[1h]

Department of Biochemistry, School of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

WILLIAM E. CONNOR, AND D. M. BRAHMANKAR

Department of Internal Medicine, College of Medicine, The University of Iowa, Iowa City, Iowa 52240

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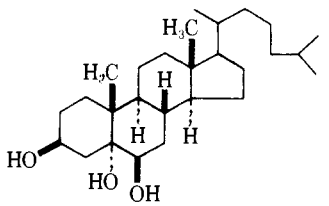
Previous studies *in vivo* demonstrated that 5α-cholestane-3β,5α,6β-triol (**1**) is a hypocholesterolemic drug in rabbits. The compound blocks the usual absorption of cholesterol into the blood and extraintestinal tissues. In this study we compare the activity *in vivo* and *in vitro* of triol **1** with selected oxo analogs and esters. These compounds are shown in influence *in vitro* a number of enzyme systems involved in cholesterol biosynthesis; an apparently new intermediate is detected. Results are discussed in terms of possible modes of action of **1** and related analogs.

5α-Cholestane-3β,5α,6β-triol (**1**)[2] exhibits marked hypocholesterolemic activity in experimental animals.[1a-c, 3] Triol **1** not only lowers the cholesterol levels in blood and liver, but also has a normalizing effect on serum phospholipid and triglyceride concentrations.[3] This compound, which is relatively nontoxic,[4] retards the usual development of aortic atherosclerosis in the cholesterol-fed rabbit[5] and lowers serum cholesterol levels from 1030 ± 149 to 43 ± 10 mg %.[1b] Tissue studies showed both liver and aorta contain negligible amounts of triol **1**, while a total of 24.35 mg is found in the intestinal mucosa (proximal to distal segments) after feeding at the usual dosage of 0.5% of the basal diet[6] for 18 weeks.[5] Investigations with [4-^{14}C]cholestane-3β,5α,6β-triol substantiated these results.[5] Sterol balance and [4-^{14}C]cholesterol absorption studies in the presence and absence of triol **1** confirmed the observation by Aramaki and coworkers[3] that the triol blocks the usual absorption of cholesterol into the blood and extraintestinal tissues.[5] These data, however, do not preclude the possibility that triol **1** may also exert an effect by inhibiting sterolgenesis in the intestinal wall.

Although triol **1** shows potential as an important hypocholesterolemic agent very little is known about structural requirements for biological activity *in vivo;*



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nothing is known about the effect *in vitro* of triol **1** or related analogs on cholesterol biosynthesis. In this report we discuss the synthesis and structure-activity relationships of triol **1** analogs *in vivo* and *in vitro* on cholesterol biosynthesis.

Results

Synthesis of Oxo Analogs and Acetate Esters.—Reaction of triol **1**[7] with Ac_2O in pyridine yielded the 3,6-diacetate derivative **2**.[8] Acid-catalyzed acetylation of diacetate **2** afforded the known triacetate ester **3**.[9] Synthesis of the 6-keto analog **4** was carried out by Fieser's method.[4] *N*-Bromosuccinimide oxidation of triol **1** afforded **4**. The 3,6-diketo analog **5** was prepared by CrO_3 oxidation of either the 6-keto compound **4** or triol **1**.[10] Selective hydrolysis of the 3,6-diacetate **2** afforded the 6-monoacetate **6**.[8] CrO_3 oxidation of acetate **6** yielded the 3-keto analog **7** of triol **1**. 5α-Cholestane-3β,5α-diol (**8**) was prepared by LAH reduction of 5α-cholestane-3β,5α,6β-triol 3-ethylcarbonate 6-methanesulfonate (**9**). Derivative **9** was synthesized by reaction of triol **1** with ethyl chloroformate in pyridine. This afforded the 3β-ethylcarbonate **10** which was subsequently converted into **9** through use of $MeSO_2Cl$. Cholestane-3β,6β-diol (**11**) was prepared according to the method of Plattner and coworkers.[11]

β-Sitosterol **12** has also been shown to exert a hypocholesterolemic effect through inhibition of cholesterol

(1) (a) D. T. Witiak, R. A. Parker, W. E. Connor, and D. M. Brahmankar, 155th National Meeting of the American Chemical Society, San Francisco, Calif, April, 1968, p N70. (b) D. T. Witiak, W. E. Connor, D. M. Brahmankar, A. Wartman, and R. Parker, *J. Clin. Invest.*, **47**, 104a (1968). (c) M. E. Dempsey, M. C. Ritter, D. T. Witiak, and R. A. Parker, *Atherosclerosis Proc. 2nd Int. Symp.*, 290–295 (1970). (d) Abstracted in part from the dissertation presented by R. A. P., July 1969, to the Graduate School of The Ohio State University. (e) Dow-Pitman-Moore Graduate Fellow 1965–1966. (f) U. S. Public Health Service Predoctoral Fellow (5-F1-GM-29,392) 1966–1969. (g) National Science Foundation Undergraduate Research Participant, Summer, 1969. (h) U. S. Public Health Service Predoctoral Fellow (1-F01-GM-42265) 1968–1970.

(2) K. Morita, German Patent 1,224,738 (1966).

(3) (a) Y. Aramaki, T. Kobayashi, Y. Imai, S. Kikuchi, T. Matsukawa, and K. Kanazawa, *J. Atheroscler. Res.*, **7**, 653 (1967). (b) Y. Imai, S. Kikuchi, T. Matsuo, Suzuoki-z, and K. Nishikawa, *ibid.*, **7**, 671 (1967).

(4) (a) K. Kaziwara, H. Yojotani, Y. Imai, S. Ogiware, and Y. Aramaki, *Jap. J. Pharmacol.*, **17**, 340 (1967). (b) S. Kikchi, Y. Imai, S. Ziro, T. Matsuo, and S. Noguchi, *J. Pharmacol. Exp. Ther.*, **159**, 399 (1968).

(5) W. E. Connor, D. Brahmankar, A. Wartman, D. T. Witiak, and R. A. Parker, unpublished data.

(6) Basal diet = 0.5% cholesterol in 2.5% peanut oil mixed with Purina Rabbit Chow.

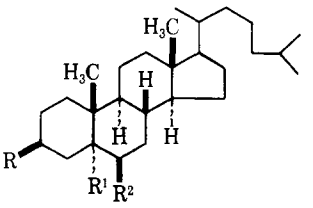
(7) L. F. Fieser and S. Rajagopalan, *J. Amer. Chem. Soc.*, **71**, 3938 (1949).

(8) B. Ellis and V. Petrow, *J. Chem. Soc.*, 1078 (1939).

(9) M. Davis and V. Petrow, *ibid.*, 2536 (1949).

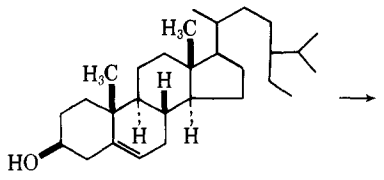
(10) V. Prelog and E. Tagmann, *Helv. Chim. Acta*, **27**, 1867 (1944).

(11) Pl. A. Plattner, H. Heusser, and M. Feurer, *ibid.*, **32**, 587 (1949).



2, R = R^2 = OAc; R^1 = OH
3, R = R^1 = R^2 = OAc
4, R = R^1 = OH; R^2 = keto
5, R = R^2 = keto; R^1 = OH
6, R = R^1 = OH; R^2 = OAc
7, R = keto; R^1 = OH; R^2 = OAc
8, R = R^1 = OH; R^2 = H
9, R = $OCO_2C_2H_5$; R^1 = OH; R^2 = OMs
10, R = $OCO_2C_2H_5$; R^1 = R^2 = OH
11, R = R^2 = OH; R^1 = H

absorption in the gut.[12] However, this compound is only one-third as effective as triol **1** in lowering serum cholesterol levels.[3] For these reasons we converted β-sitosterol (**12**) into the corresponding 3β,5α,6β-triol derivative **13**. This derivative, 5α-stigmastane-3β-5α,6β-triol (**13**) is prepared from purified β-sitosterol using a procedure similar to the preparation of triol **1**.



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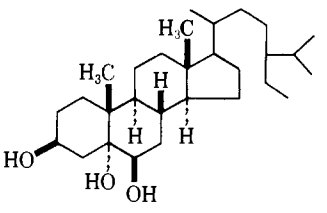

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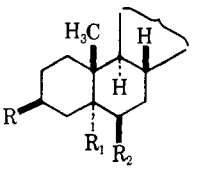
Biological Evaluation *in Vivo* **of Selected Oxo Analogs.**—Relatively large amounts of compound are required for biological evaluation *in vivo*. For these reasons only a few selected analogs of triol **1** were investigated (Table I). Compounds chosen complement structure-activity studies reported in the literature.[3] Activity *in vivo* was determined by the same method used to evaluate the parent triol **1**.[5] After 15-days ingestion of a diet containing 0.5% cholesterol and 0.5% drug, the rabbits were sacrificed and the serum cholesterol concentration was determined. Experiments with control rabbits (fed only cholesterol) and rabbits fed cholesterol and triol **1** were repeated many times; results with all animals were very consistent. Since large amounts of compound are required, fewer animals were used for evaluation *in vivo* of triol **1** analogs. The triacetate **3**, 6-keto analog **4**, and the 3,6-diketo analog **5** exhibited only a slight hypocholesterolemic effect. Comparison with triol **1** activity revealed these compounds to be considerably less active. Alternatively, the 3β,5α-diol analog **8** showed a significant hypocholesterolemic effect in 2 rabbits.

We had anticipated that 5α-stigmastane-3β,5α,6β-triol (**13**) might also exhibit excellent hypocholesterolemic activity like triol **1**. The results with triol **13** are illustrated in Figure 1. For the first 7 days after feeding Purina Chow, a basal diet containing 0.5% cholesterol[6] was fed to the rabbits; during this period the serum cholesterol level increased from 50 mg % to 650 mg %. Subsequent administration of the basal diet[6] plus 0.5% 5α-stigmastane-3β,5α,6β-triol (**13**) for 2 weeks only increased the serum cholesterol an additional 150 mg %. When triol **13** was removed from the diet during the last week of the study the serum cholesterol again dramatically increased to 1100 mg %. These data show triol **13** does retard development of hypercholesterolemia in the cholesterol-fed rabbit. However, the compound is considerably less active than triol **1**. Under similar experimental conditions triol **1** not only retards development of hypercholesterolemia, but actually lowers serum cholesterol to nearly normal levels.[5]

(12) (a) L. Swell, E. C. Trout, Jr., H. Field, Jr., and C. R. Treadwell, *J. Biol. Chem.*, **234**, 2286 (1959). (b) T. Gerson, F. B. Shorland, and G. G. Duncklev, *Biochem. J.*, **96**, 399 (1965).

TABLE I

ACTIVITY *in Vivo* OF 5α-CHOLESTANE-3β,5α,6β-TRIOL (1) AND SELECTED ANALOGS ON SERUM CHOLESTEROL LEVELS



Compd	No. rabbits tested	Init serum cholesterol, mg %	Terminal serum cholesterol,[a] mg %
Control (cholesterol but no drug fed)	27	37	971 ± 125[b]
1, R = R^1 = R^2 = OH	17	31	63 ± 15
3, R = R^1 = R^2 = OAc	4	23	655 ± 239
4, R = R^1 = OH; R^2 = keto	5	54	631 ± 198
5, R = R^2 = keto, R^1 = OH	2	43	553 ± 47
8,[c] R = R^1 = OH; R^2 = H	1	17	53[d]
		21	235[e]

[a] After feeding equal amts (0.5% each) of cholesterol and drug for 3 weeks. [b] Standard error. [c] Two rabbits were used for these preliminary results. Since only 2 rabbits were used we prefer to report these results with each rabbit rather than as a mean value. [d] After feeding cholesterol alone to this rabbit for 3 weeks the serum cholesterol rose to 446 mg %. [e] After feeding cholesterol alone to this rabbit for 3 weeks the serum cholesterol rose to 1034 mg %.

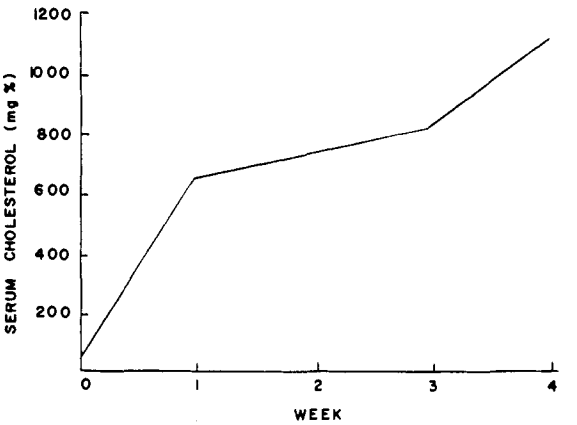
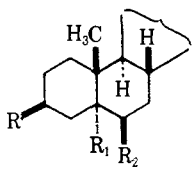


Figure 1.—The effect of 5α-stigmastane-3β,5α,6β-triol (**13**) on serum cholesterol levels of cholesterol-fed rabbits. Dietary periods: 0 week = Purina Chow; 1 week = 0.5% cholesterol with Purina Chow; 2 and 3 weeks = 0.5% cholesterol and 0.5% stigmastane-3β,5α,6β-triol (**13**) with Purina Chow; 4 weeks = 0.5% cholesterol with Purina Chow. Each value represents the average results of 6 rabbits.

Inhibition *in Vitro* of Cholesterol Biosynthesis by Selected Oxo Analogs.—The effects of 5α-cholestane-3β,5α,6β-triol (**1**) and selected oxo and desoxy analogs added *in vitro* on the incorporation of [2-^{14}C]acetate and [2-^{14}C]mevalonate into nonsaponifiable products are found in Table II. The data presented in Table II

TABLE II

EFFECTS OF 5α-CHOLESTANE-3β,5α,6β-TRIOL (**1**) AND SELECTED OXO AND DESOXY ANALOGS ADDED *in Vitro* ON THE INCORPORATION OF [2-^{14}C]ACETATE AND [2-^{14}C]MEVALONATE INTO NONSAPONIFIABLE PRODUCTS



Compd[a]	% inhib of ^{14}C incorp into cholesterol from— Acetate[b]	Mevalonate[b]	Nonsaponifiable products containing 50% or more of the radioactivity originally as acetate or mevalonate[c]
1, R = R^1 = R^2 = OH	98.0[d]	97.2[e]	C-29–30
3, R = R^1 = R^2 = OAc	4.6	(−4.7)	C-27
5, R = R^2 = keto; R^1 = OH		90.0[f]	C-28–30
6, R = R^1 = OH; R^2 = OAc	38.4	19.7	C-27
8, R = R^1 = OH; R^2 = H	57.2[g]	66.1[h]	C-29–30
11, R = R^2 = OH; R^1 = H	71.5[i]	57.2[i]	C-27
13, stigmastanetriol		50.0[f]	C-28–30
Cholesterol	(−410)[g]		C-28–30
Cholesterol		(−124)[h]	C-27
Cholestanol	(−2.5)	(−2.6)	C-27
5α-Cholestane	(−2.5)	0.5	C-27

[a] Analogs were dissolved in propylene glycol or dioxane. [b] Incubations of [2-^{14}C]acetate and [2-^{14}C]mevalonate with rat liver preps and chromatographic separation and identification of nonsaponifiable products were as given with Figures 2 and 3 except that the analog concn in each incubation is 60 μ*M*. Per cent inhibition or activation (−) data listed are the average value with a standard error no greater than ±0.4%. [c] The nonsaponifiable products which accumulated in the presence of the analog and which contained 50% or more of the radioactivity originally present as acetate or mevalonate are indicated (C-28–30 = squalene plus 28–30-C atom sterols; C-27 = cholesterol and other 27 C atom sterols). [d] In the absence of the analog greater than 50% of the nonsaponifiable radioactivity was present as 27 C atom sterols (see also Figures 2 and 3). [e] In the absence of analog greater than 50% of the nonsaponifiable radioactivity was present as 27–28 C atom sterols (see also Figures 2 and 3). [f] In the absence of analog 40% of the nonsaponifiable radioactivity was present as 27-C atom sterols and the remaining radioactivity was distributed among 28–30-C atom compds. [g] In the absence of the analog greater than 50% of the nonsaponifiable radioactivity was present as squalene and 29–30-C atom sterols. [h] In the absence of analog 45% of the nonsaponifiable radioactivity distributed among the 28–30-C atom compds. [i] In the absence of analog greater than 50% of the nonsaponifiable radioactivity was present as 27-C atom sterols.

and Figure 2 show that 50% inhibition of the conversion of acetate and mevalonate to cholesterol occurs in the presence of approximately 5 μ*M* triol **1**; the inhibition is complete at 50 μ*M* triol **1**. A related finding is that there is no inhibition by the triol and usually a slight activation (15%), of the conversion of acetate and mevalonate into nonsaponifiable compounds other than cholesterol. Stigmastanetriol (**13**) also inhibited conversion of mevalonate to cholesterol *in vitro*, but with lower potency than triol **1**. In studies similar to those outlined in the conditions with Figure 2, triol **1** was found to inhibit the conversion of acetate into cholesterol by rat intestinal slices and mucosal preparations. The other analogs have not been tested for effects in the intestinal preparation. Findings similar to those just described are also obtained with squalene as substrate for the enzyme system.

Inhibition of cholesterol synthesis by the triol results in the accumulation of a compound which migrates during silicic acid column chromatography as a 29–30-C atom sterol slightly more polar than lanosterol (Figure 3). This compound is readily converted into cholesterol by an uninhibited enzyme preparation. Addition of Δ^{5,7}-cholestadien-3β-ol to a solution containing the unidentified compound followed by epiperoxide formation[13] and chromatography afforded approximately 60% of the radiolabel in a mixture with Δ^6-cholesten-3β-ol-5α,8α-epiperoxide. It seems likely the unidentified intermediate contains a conjugated double bond system; such systems, like the Δ^{5,7} system, are readily converted into an epiperoxide derivative.[13] However, this unidentified compound did not migrate with 4,4-dimethyl-Δ^{5,7}-cholestadien-3β-ol.[14] One possible structure of the compound is 4,4-dimethyl-Δ^{8,14}-cholestadiene-3β-ol, a product of lanosterol demethylation.[15] In contrast to triol **1**, stigmastanetriol (**13**) did not cause formation of the 29–30 carbon atom sterol, but did result in accumulation of an unidentified 28-carbon atom sterol. None of the analogs tested caused accumulation of Δ^{5,24}-cholestadien-3β-ol, as when MER-29 (1-[*p*-(β-diethylaminoethoxy)phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol) and some other inhibitors of cholesterol synthesis are employed.[16,17]

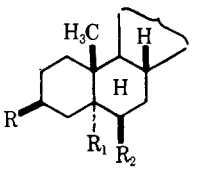
The triacetate derivative **3** of triol **1** does not inhibit either [2-^{14}C]acetate or [2-^{14}C]mevalonate incorporation into cholesterol (Table II). While the 3,6-diketo analog **5** is an effective inhibitor of [2-^{14}C]mevalonate incorporation into nonsaponifiable products the 6-acetoxy compound **6** is considerably less potent than the parent triol **1**. The 3β,5α- and 3β,6β-diols (**8** and **11**, respectively) show more activity than either of the acetoxy esters **3** and **6**. Unlike the situation *in vivo*, cholesterol exhibits a marked stimulation of radiolabeled acetate and mevalonate incorporation into nonsaponifiable products. Cholestanol and 5α-cholestane show no significant effect on acetate and mevalonate incorporation into cholesterol (C-27 sterols) *in vitro*.

The effects of triol **1** and selected oxo and desoxy analogs added *in vitro* on two semipurified liver enzymes catalyzing steps in cholesterol synthesis are found in Table III. The results in Figure 4 also show the effect of varying concentrations of triol **1** on these two enzymes, namely, Δ^7-sterol Δ^5-dehydrogenase and Δ^{5,7}-sterol Δ^7-reductase.[13,18] Triol **1** inhibits both of these enzymes. It is approximately twice as effective against the reductase as against the dehydrogenase at a concentration of approximately 50 μ*M*. The triacetate derivative **3**, which has no effect on acetate or mevalonate incorporation into nonsaponifiable sterols is a potent activator

(13) M. E. Dempsey, *Methods Enzymol.*, **15**, 501 (1969).
(14) A gift from Drs. E. Paoletti and A. Fiecchi, University of Milan, Milan, Italy.
(15) L. Canonica, A. Fiecchi, M. G. Kienle, A. Scala, G. Galli, E. G. Paoletti, and R. Paoletti, *J. Amer. Chem. Soc.*, **90**, 6532 (1968).
(16) M. E. Dempsey, *Ann. N. Y. Acad Sci.*, **148**, 631 (1968).
(17) M. E. Dempsey, *Progr. Biochem. Pharmacol.*, **3**, 21 (1967).
(18) M. C. Ritter and M. E. Dempsey, *Biochem. Biophys. Res. Commun.*, **38**, 921 (1970).

TABLE III

EFFECTS OF 5α-CHOLESTANE-3β,5α,6β-TRIOL (1) AND SELECTED OXO AND DESOXY ANALOGS ADDED *in Vitro* ON Δ⁷-STEROL Δ⁵-DEHYDROGENASE AND Δ⁵,⁷-STEROL Δ⁷-REDUCTASE



Compd[a]	Substrate: Δ⁷-Cholestenol,[b] % inhib (−) or activation (+)/μM of analog tested	Substrate: Δ⁵,⁷ Cholestadienol,[c] % inhib (−) or activation (+)/μM of analog tested
1, R = R¹ = R² = OH	−11/52; −23/121	−25/67; −35/90
3, R = R¹ = R² = OAc	+31/58	+38/58
4, R = R¹ = OH; R² = keto	−14/63	+26/63
5, R = R¹ = OH; R² = keto	−5/40	−5/20
6, R = R¹ = OH; R² = OAc	−9/68	−24/68
7, R = keto, R¹ = OH; R² = OAc	−7/60	+55/60
8, R = R¹ = OH; R² = H	−9/56; −21/121	−31/70
10, R = $OCO_2C_2H_5$; R¹ = R² = OH	0/68	−1/68
11, R = R² = OH; R¹ = H	−8/53; −11/121	−5/67
13, stigmastanetriol	−7/51; −18/103	−20/61
Cholesterol	−15/52; −20/104	−50/34; −65/67
Cholestanol	0/28; −4/77	−37/77
5α-Cholestane	+2/86	+2/86
Δ⁴-Cholesten-3-one	−5/20; +4/60	0/10; +10/30; +18/60

[a] Analogs were dissolved in propylene glycol or dioxane. [b] Conversion of Δ⁷-cholesterol into Δ⁵,⁷-cholestadienol was assayed by the uv absorption technique [M. E. Dempsey, *Methods Enzymol.*, **15**, 501 (1969)]. Concns of the constituents of each incubation medium (total vol, 1.3 ml) were 0.1 *M* phosphate buffer, pH 7.35, 1 m*M* NAD, 30 μ*M* Δ⁷-cholestenol, 0.1 n*M* *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride [D. Dvornik, M. Kraml, and J. F. Bagli, *Biochemistry*, **5**, 1060 (1966)] analog as indicated, 3 mg of microsomal enzyme protein, and 15 mg of activator protein. Incubations were for 45 min at 37° under O_2. [c] Conversion of [4-¹⁴C] Δ⁵,⁷-cholestadienol into [4-¹⁴C]cholesterol was assayed by the dibromide derivative technique (see footnote *b*). Concns of the constituents of each incubation medium (total vol, 2.2 ml) are 0.1 *M* phosphate buffer, pH 7.35, 1 m*M* NADPH, 20 μ*M* [4-¹⁴C]Δ⁵,⁷-cholestadienol analog as indicated, and microsomal enzyme and activator protein as for Δ⁷-cholestenol incubations (see footnote *b*). Incubations were for 45 min at 37° under N_2.

of both these semipurified enzyme systems. The only other analogs tested, which activate the reductase, are the 6-keto analog **4** and the 3-keto analog as its 6-acetoxy derivative **7**. These two compounds as well as the 6-acetoxy derivative **6** of the triol, the 3β,5α- and 3β,6β-diols (**8** and **11**, respectively) and stigmastanetriol **13** all inhibit the Δ⁷ → Δ⁵,⁷ sterol conversion. Compounds **6**, **8**, **11**, and **13** also significantly inhibit conversion of Δ⁵,⁷-cholestadien-3β-ol into cholesterol. Interestingly, esterification of the 3β-hydroxyl group (**10**) of triol **1** renders the compound inactive against either the reductase or the dehydrogenase enzyme. Cholesterol also is an inhibitor of the semipurified reductase and dehydrogenase enzymes. Reduction of the Δ⁵ bond of cholesterol (*i.e.*, cholestanol) yields an inhibitor of only the reductase enzyme. 5α-Cholestane is essentially inactive against both enzymes, while Δ⁴-cholesten-3-one (shown here to be a metabolic product of diol **8**) is a stimulator at concentrations above 30 μ*M*.

Experiments performed with 4-¹⁴C-labeled triol **1** and the 3β,5α-diol **8** demonstrated that neither **1** nor **8** is converted into cholesterol by rat liver preparations capable of active cholesterol synthesis. However,

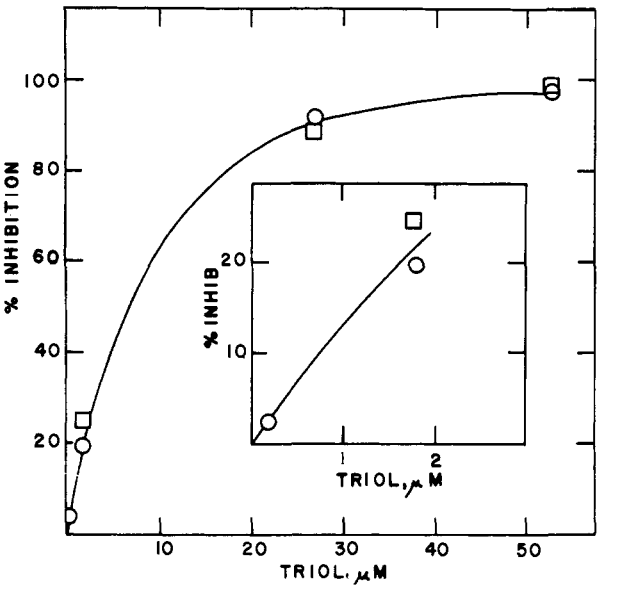


Figure 2.—Effects of varying concns of triol (**1**) on the enzymic conversion of acetate and mevalonate to cholesterol: (○—○) conversion of [2-¹⁴C]mevalonate into cholesterol; (□—□) conversion of [2-¹⁴C]acetate into cholesterol. Concns of the constituents of each incubation medium (total vol 1.35 ml) were 0.1 *M* phosphate buffer, pH 7.35, 2.3 × 10⁵ dpm of Na [2-¹⁴C]-acetate (2.0 mCi/mmole) or 3.9 × 10⁵ dpm of [2-¹⁴C]mevalonate (3.1 mCi/mmole), 0.8 m*M* NADPH, NADP, and NAD, and 5 m*M* ATP, 5 m*M* $MgCl_2$, cholestane-triol (**1**), as indicated, and 22.5 mg of protein from the 500*g* supernatant fraction of a rat liver homogenate. Incubations were for 30 min at 37° under O_2. Conversions into nonsaponifiable compounds, sterols, and cholesterol were determined as described in detail elsewhere.[13,16]

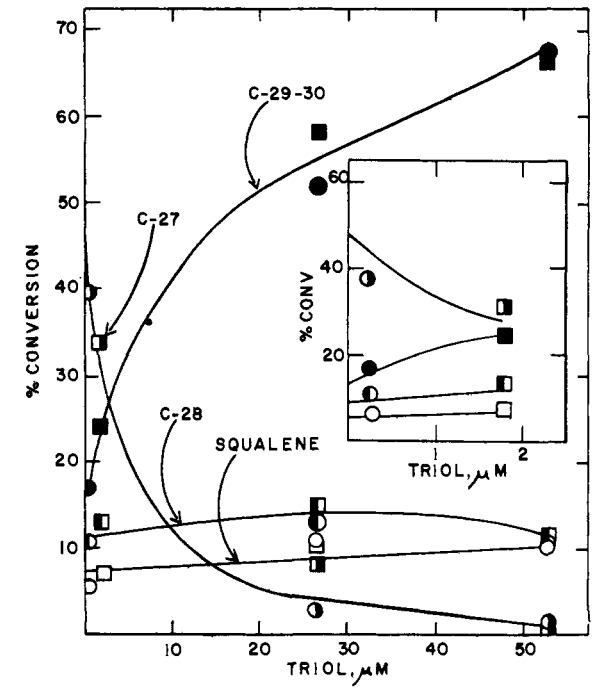


Figure 3.—Accumulation of 29-30-C atom sterols *in vitro* in the presence of varying concentrations of triol (**1**). Conversion of [2-¹⁴C]mevalonate into squalene (○—○), to 29-30-C atom sterol (●—●), to 28 C atom sterols (◐—◐), and to 27-C atom sterols including cholesterol (◑—◑). Conversion of [2-¹⁴C]-acetate to squalene (□—□), to 29-30-C atom sterols (■—■), to 28-C atom sterols (◧—◧), and 27-C atom sterols including cholesterol (◨—◨). Other conditions were as given with Figure 2.

both **1** and **8** are metabolized *in vitro*. The metabolites of both sterols have the spectral characteristics of the

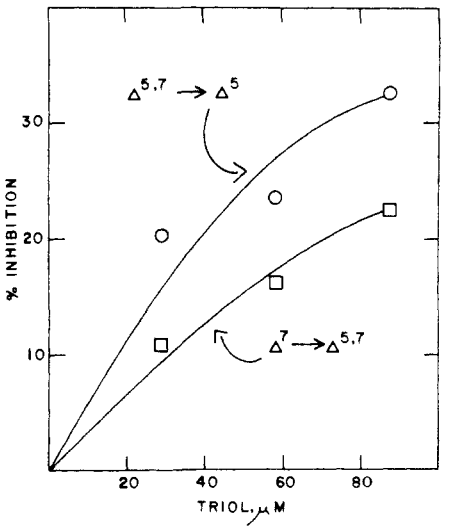


Figure 4.—Effects of varying concentrations of cholestane-3β,5α,6β-triol (1) on purified Δ7-sterol Δ5-dehydrogenase and Δ5,7-sterol Δ7-reductase of rat liver: (O—O) conversion of [4-14C]Δ5,7-cholestadienol into [4-14C]cholesterol; (□—□) conversion of [2,4-3H] Δ7-cholestenol into [2,4-3H] Δ5,7-cholestadienol. Concns of the constituents of each incubation medium (total vol, 2.45 ml) were 0.1 *M* phosphate buffer, pH 7.35, 1 m*M* NaDPH and 31.8 μ*M* [4-14C] Δ5,7-cholestadienol or 1 m*M* NAD, 31.7 μ*M* [2,4-3H] Δ7-cholestenol, and 0.1 n*M* *trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride, triol **1** as indicated, and 2.4 mg of purified enzyme system protein and 30 mg of activator protein. Incubations were for 45 min at 37° under N_2 (Δ5,7→Δ5) or O_2 (Δ7→Δ5,7). Prepn of the purified enzyme system and assays for enzymic activities were performed as described elsewhere.[13]

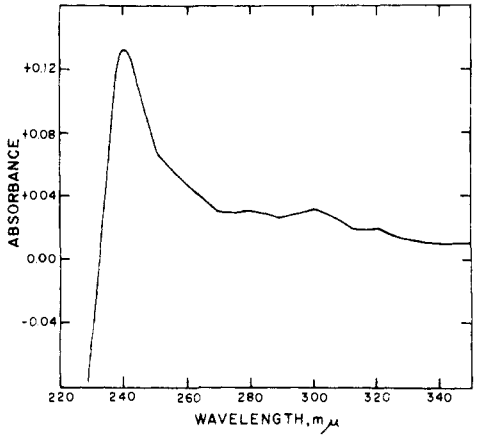


Figure 5.—Difference spectrum showing enzymic conversion of diol **8** to Δ4-cholesten-3-one. Concns of the constituents of the incubation medium (total vol, 4.8 ml) were 0.1 *M* phosphate buffer, pH 7.35, 1 m*M* NADPH, 1 m*M* NADH, 1040 μ*M* diol **8**, 10 mg of microsomal enzyme, and 70 mg of activator protein. Incubation was for 45 min at 37° under O_2. The uv spectrum of the cyclohexane extract of this incubation was compared with that of a parallel incubation identical but without diol **8**.

Δ4-3-ketone system;[19] a λ_{max} at 240 mμ (Figure 5 for the product of diol **8**) is characteristic of such a function. The metabolic product of diol **8** also migrates during silicic acid chromatography with unlabeled Δ4-cholesten-3-one. Based on the molar absorptivity coefficient for Δ4-cholesten-3-one[19] and the original specific radioactivity of diol **8**, the maximum conversion *in vitro* of diol **8** into Δ4-cholesten-3-one is 2%. An even lower per cent of radioactivity originally present in triol **1** or diol **8** was detected in a highly polar compound with the chromatographic characteristics of a bile acid. Its low level precluded further identification.

Discussion

The 3,6-diformate derivative of triol **1** has previously been shown by Aramaki and coworkers[3] to have hypocholesterolemic activity. Other mono- and disubstituted esters at positions 3 and 6 in triol **1** are also active *in vivo*.[3] One possibility for the observed inactivity of the triacetate **3** is that the 5α ester does not undergo hydrolysis in the gut. This ester is also resistant to alkaline-catalyzed hydrolysis. While the 5α-OH seems to be most important for hypocholesterolemic activity *in vivo* the presence of this group is not the only function contributing to the effectiveness of the compound. Preliminary results employing the 3β,5α-diol **8** suggest the 6-OH is not important for biological activity *in vivo*. However, it is also apparent that minor structural modifications not related to the 3β- and 5α-OH may render an analog less potent or completely inactive. For example, conversion of the 6-OH function into a ketone renders the compound inactive. The 3,6-diketo analog **5** has only slight activity. Insertion of an Et group in the 24 position, a position far removed from the A–B ring system, also affords an analog (**13**) which is considerably less effective than the original triol **1**.

Previous studies[1b,3,5] *in vivo* show that triol **1** blocks the usual absorption of cholesterol into the blood and extraintestinal tissues. While this probably represents a major mechanism, triol **1** may also be operating in part by other mechanisms. Our data *in vitro* suggest that triol **1** may be working concomitantly by inhibiting sterolgenesis in the intestinal wall. It is known that substantial amounts of cholesterol are synthesized by the intestine.[20] Imai and coworkers[3b] have shown that triol **1** administered orally enhances cholesterolgensis in both liver slices and the liver of intact rats. For these reasons they suggest the compound does not exert its activity by inhibiting sterolgenesis. As we pointed out previously,[5] triol **1** is not absorbed into the extraintestinal tissues to any large degree. However, as shown here (Figures 2 and 3) 50% inhibition of cholesterol synthesis *in vitro* occurs with approximately 5 μ*M* triol **1**. It is probable that triol **1** does not block cholesterolgenesis in the liver, but some inhibition cannot be excluded in view of the low level required for activity *in vitro*. The effect of increased hepatic cholesterolgenesis *in vivo* is likely a reflection of maintenance of homeostasis of the cholesterol level. In this regard we noted activation of sterol (other than cholesterol) synthesis *in vitro* with triol **1**.

Although we have shown that triol **1** does inhibit cholesterolgenesis in isolated intestinal tissue, most of our work, designed to probe structural requirements for activity *in vitro*, was carried out in rat liver homogenate preparations. Such preparations were employed because the effect of the compounds could more easily be compared with sterolgenesis inhibitory studies published for other compounds. Further, the methods

(19) L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Co., New York, N. Y., 1959, pp 18–21.

(20) (a) J. M. Dietschy and M. D. Siperstein, *J. Lipid Res.*, **8**, 97 (1967). (b) J. Dupont, K. S. Atkinson, and L. Smith, *Steroids*, **10**, 1 (1967).

for preparation of the semipurified enzymes, Δ^7-sterol Δ^5-dehydrogenase and $\Delta^{5,7}$ sterol-Δ^7-reductase, are well established.[13, 16-18]

In rat liver homogenate and intestinal preparations triol **1** is an effective inhibitor of acetate, mevalonate, and squalene incorporation into nonsaponifiable products. The triacetate derivative **3**, which is inactive *in vivo*, is also inactive in rat liver homogenate preparations. Similarly, the 3β,5α-diol (**8**) inhibits sterolgenesis *in vivo* and *in vitro*. The only discrepancy in effects seen *in vivo* and *in vitro* is that the 3,6-diketo compound **5** is active *in vitro* but not *in vivo*. Observations that cholestanol and 5α-cholestane are not active *in vitro* show that hydroxylation at the site of the 5 and 6 positions contributes to the compounds' ability to bind to the enzymes involved in cholesterol biosynthesis. Our results showing cholesterol activates acetate and mevalonate conversion into cholesterol *in vitro* must reflect a direct effect on the enzymes, either an activation or protection from deactivation occurring in the control incubation. These results are not related to the physiological control mechanisms observed *in vivo*. These feedback mechanisms cannot be demonstrated *in vitro;* they require cholesterol feeding or starvation and then changes are seen in liver preparations *in vitro*.

Multiple mechanisms of action of triol **1** and related analogs are substantiated by noting the results obtained with the semipurified enzyme systems. Extrapolation of results listed in Table III obtained *in vitro* to considerations of activity *in vivo* is precluded at this time. However, some structural requirements for activity *in vitro* on Δ^7-sterol Δ^5-dehydrogenase and $\Delta^{5,7}$-sterol Δ^7-reductase are evident. First, blocking all three OH functions of triol **1** in the form of acetate esters converts the compound from an inhibitor of these two enzyme systems to a stimulator of both systems. Keto derivatives **4** and **7** only stimulate the reductase system. These compounds slightly inhibit Δ^7-sterol Δ^5-dehydrogenase. Apparently, the 3β-OH is important for blocking activity since conversion into the β-ethyl carbonate **10** renders the compound inactive. Δ^4-Cholesten-3-one is the only other compound exhibiting stimulation of the reductase enzyme, but at elevated concentrations. Except for cholestane itself, most other structural modifications afford compounds which inhibit these enzymes. These observations are far reaching in light of the possible interest in using triol **1** clinically. It is possible that like triparanol use of this compound may cause accumulation of other sterols. This is of particular significance since these studies show a major action of triol **1** and related compounds to be occurring between squalene and cholesterol and that there is an accumulation *in vitro* of an apparently new intermediate in cholesterol biosynthesis. Investigations are in progress to identify the intermediate and determine the significance of its accumulation.

A final point should be raised when considering the results obtained *in vitro* with those observed *in vivo*. Aramaki and coworkers[3] report that triol **1** is metabolized at a much higher rate than is cholesterol and that one of the products results from apparent oxidative removal of C-25, -26, and -27 and conversion of C-24 into a CO_2H group. We also observed[5] radiolabel in a bile acid fraction of feces after feeding 4-^{14}C-labeled triol **1**. In liver homogenate preparations *in vitro* a major metabolite of triol **1** appears to result from loss of H_2O and oxidation of the 3β-OH group to a ketone, thereby affording a 6β-hydroxy-3-ket-4-ene derivative. Although these data suggest a similar dehydration and oxidation may also take place *in vivo*, further work is required to substantiate this proposal. A compound with the properties of a bile acid was also detected *in vitro* similar to the findings *in vivo*.

Experimental Section[21]

5α-Cholestane-3β,5α,6β-triol (1) was prepd by the method of Fieser and Rajogopalan.[7] In order to prepare the large amounts of triol needed the following modified procedure was used. Cholesterol (200 g, 0.52 mole) and 2 l. of 90% HCO_2H were heated on a steam bath with occasional stirring for approx 1 hr. An oily layer of the corresponding formate ester sepd. The reaction mixture was cooled on an ice bath with agitation in order to obtain a fine white ppt. H_2O_2 (200 ml of 30% soln, 1.47 moles) was added and the mixt was shaken occasionally and allowed to stand at room temp overnight. Care must be taken to prevent the reaction from heating above 45°. The reaction was allowed to continue until a clear, faintly blue soln was obtained. Boiling H_2O (3.5 l.) was added to the reaction with constant stirring to decompose excess H_2O_2 and ppt the reaction product. The mixt was allowed to cool in a refrigerator and the white ppt was collected and dried. The ppt was transferred to a 6-l. flask and heated to reflux with 5 l. of MeOH. Aq NaOH (25%, 200 ml) was slowly added to the refluxing soln. After addition, the reaction mixture was refluxed for an additional 1.5–2 hr. Concd HCl was added with stirring to neutralize the soln. Upon cooling triol **1** crystd as white needles. Recrystn from MeOH afforded pure 5α-cholestane-3β,5α,6β-triol. The mother liquor was heated and H_2O was added to the cloud point to obtain more triol **1**. The combined cryst triol totaled 200 g (92%), mp 242–244°, lit.[7] mp 244°.

5α-Cholestane-3β,5α,6β-triol 3,6-diacetate (2) was prepd from triol **1** by the usual methods employing excess Ac_2O in pyridine at room temp, mp 165–166°, lit.[9] mp 166°.

5α-Cholestane-3β,5α,6β-triol 3,5,6-Triacetate (3).—The diacetate **2** was converted into the triacetate **3** by an acid-catalyzed acetylation as described by Davis and Petrow,[9] mp 148–149°, lit.[9] mp 149°.

5α-Cholestane-3β,5α-diol-6-one (4).—Triol **1** was oxidized with NBS affording the corresponding 6-keto analog **4** according to the method of Fieser and Rajogopalan,[7] mp 232–233°, lit.[7] mp 232°.

5α-Cholestane-3,6-dione-5α-ol (5).—Triol **1** was converted to the 3,6-dione **5** as described by Prelog and Tagmann,[10] mp 235° dec, lit.[10] 232° dec.

5α-Cholestane-3β,5α,6β-triol 6-acetate (6) was prepd by selective hydrolysis of the 3,6-diacetate **2** as described by Ellis and Petrow,[8] mp 143–144°, lit.[8] mp 144°.

5α-Cholestane-5α,6β-diol-3-one 6-acetate (7) was prepd by the method of Ellis and Petrow,[8] mp 161–162°, lit.[8] mp 161–162°.

5α-Cholestane-3β,5α-diol (8).—5α-Cholestane-3β,5α,6β-triol 3-ethylcarbonate 6-methanesulfonate (2.00 g, 3.5×10^{-3} mole, **9**) in 75 ml of Et_2O was added dropwise with stirring to LAH (2.00 g, 5.0×10^{-2} mole) at 0°. The reaction was allowed to stir at room temp overnight. The reaction mixt was poured into ice H_2O and extd (Et_2O). The Et_2O layer was washed with H_2O, dried (Na_2SO_4), filtered, and removed under reduced pressure affording 1.40 g white cryst residue. Recrystn from Me_2CO–MeOH (1:1) gave 1.35 g (95%) of **8**, mp 224–225°, lit.[22] mp 225°.

5α-Cholestane-3β,5α,6β-triol 3-Ethylcarbonate 6-Mesylate (9).—Ethyl carbonate **10** (36.0 g, 9.5×10^{-2} mole) in 120 ml of dry pyridine was cooled on an ice bath. MsCl (60.0 g, 0.525 mole) in 50 ml of pyridine added dropwise with stirring and the mixt was allowed to stand at room temp overnight. It was poured into ice H_2O and extd (Et_2O). The dried (Na_2SO_4) Et_2O layer was evapd under reduced pressure affording 36.0 g of cryst prod-

(21) Melting points are corrected and are taken using a Thomas-Hoover melting point apparatus. Elemental analyses are performed by Clark Microanalytical Labs., Urbana, Ill.

(22) Pl. A. Plattner, Th. Petrzilka, and W. Lang, *Helv. Chim. Acta*, **27**, 513 (1944).

uct. Recrystn from Me_2CO afforded 29.0 g (70%) of **9**, mp 174–176° dec. For instability reasons **9** was not further purified and subjected to elemental analysis.

5α-Cholestane-3β,5α,6β-triol 3-Ethyl Carbonate (10).—Triol **1** (40.0 g, 9.5×10^{-2} mole) in 400 ml of pyridine was cooled on an ice bath. Ethyl chloroformate (88.0 g, 0.81 mole) was added dropwise with stirring. The reaction mixt was allowed to stand at room temp for 6 hr, poured into H_2O, and extd with Et_2O. The dried (Na_2SO_4) Et_2O layer was evapd under reduced pressure. Recrystn from MeOH–Me_2CO (1:1) afforded 40 g (85%) of **10**, mp 185–188°, lit.[23] mp 188°.

5α-Cholestane-3β,6β-diol (11) was prepd according to the method of Plattner and coworkers[11] affording crystals, mp 192–193°, lit.[11] mp 192°.

5α-Stigmastane-3β,5α,6β-triol (13).—β-Sitosterol (**12**) (50 g, 0.124 mole) suspended in 500 ml of 90% HCO_2H was heated on a steam bath with occasional stirring for 1 hr. The mixt was allowed to cool to room temp and 80 ml of 30% H_2O_2 was added. The reaction mixt was stirred for 72 hr at room temp. Boiling H_2O (3 l.) was added with stirring; the mixt was cooled and filtered. The dried filtrate was heated to reflux in 3 l. of MeOH. An aq soln of NaOH (25%, 80 ml) was added and the mixt was refluxed for 1 hr, neutralized with 10% HCl, and allowed to cool to room temp. Compd **13** crystallized affording white needles. Recrystn from MeOH afforded 54 g (94%) of **13**, mp 242–245°, lit.[24] mp 248–250°. *Anal.* ($C_{29}H_{52}O_3$) C, H.

Biological Studies *in Vivo*.—Rabbits were fed Purina rabbit laboratory chow for at least 3 weeks. The rabbits were then fed equal amts of the drug under study and 0.5% cholesterol in 2.5% peanut oil mixed with the same purina chow for 3 weeks. With active compds a final dietary period consisting of only 0.5% cholesterol in 2.5% peanut oil was administered with purina chow for an additional 3 weeks. At the beginning and end of each dietary regimen, body wt of the rabbit was measured and blood samples were collected for serum cholesterol determination which was measured by the method of Abell, *et al.*[25]

Biological studies *in vitro* were carried out according to methods previously reported by Dempsey and coworkers.[13,16–18]

Acknowledgment.—We are grateful to the National Institutes of Health for support of this work through Grants HE-12740, HE-8364, and HE-6314 from the National Heart Institute. This investigation was supported (in part) by National Institutes of Health Research Grant No. FR-00328 from the Division of Research Facilities and Resources.

(23) L. F. Fieser, J. C. Hortz, M. W. Klohs, M. A. Romero, and T. Ulne, *J. Amer. Chem. Soc.*, **74**, 3309 (1952).

(24) D. H. Coffey, I. M. Heilbron, and F. S. Spring, *J. Chem. Soc.*, 738 (1936).

(25) L. L. Abell, B. B. Levy, B. B. Brodie, and F. E. Kendell, *J. Biol. Chem.*, **195**, 357 (1952).

Agents for Alkylating Steroid Hormone Receptors. 2. 16α-Substituted Progesterone Derivatives[1]

A. J. SOLO* AND JOHN O. GARDNER

Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14214

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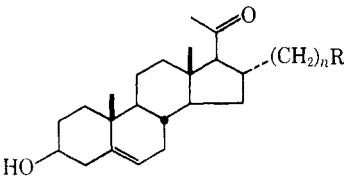
Derivatives of 16α-propyl-, 16α-butyl, and 16α-pentylprogesterone were synthesized and tested for Clauberg activity. Derivatives of the 3-carbon side chain included the ω alcohol, mesylate, tosylate, bromoacetate, and *p*-fluorosulfonylbenzoate. The 4-carbon side chain was derivatized as the ω alcohol, mesylate, and bromoacetate. Derivatives of the *n*-pentyl chain included ω alcohol, mesylate, tosylate, bromoacetate, *p*-fluorosulfonylbenzoate, toluate, and bromide.

In a search for an agent capable of selectively alkylating the Clauberg receptor, we recently investigated a series of diazo ketones derived from esters of 17α-hydroxyprogesterone.[2] In a continuation of that search, we have prepared and had assayed a series of compounds containing functional groups linked by *n*-alkyl side chains to the 16α position of progesterone.

Chemistry.—16-Dehydropregnenolone acetate was treated with the Grignard reagent prepared from 3-*tert*-butoxy-1-bromopropane.[3] Oppenauer oxidation of the resulting 3β-hydroxy-16α-(3-*tert*-butoxypropyl)pregn-5-en-20-one (I) afforded 16α-(3-*tert*-butoxypropyl)-pregn-4-ene-3,20-dione (II). The *tert*-butyl group of II was removed by treatment with CF_3CO_2H to afford alcohol III. The latter compound was converted into mesylate IV, bromoacetate V, tosylate VI, and *p*-fluorosulfonylbenzoate VII by the usual methods.

Grignard reagents prepared from 4-bromobutene and 5-bromopentene reacted with 16-dehydropregnenolone acetate to afford 3β-hydroxy-16α-(3-butenyl)pregn-5-en-20-one (VIII) and 3β-hydroxy-16α-(4-pentenyl)-pregn-5-en-20-one (IX), respectively.[3] Oppenauer oxidation of VIII produced X, and XI was similarly formed from IX. These compounds were each hydroborated and then oxidized by alkaline H_2O_2 to produce 16α-(4-hydroxybutyl)pregn-4-ene-3,20-dione (XII) and 16α-(5-hydroxypentyl)pregn-4-ene-3,20-dione (XIII). Alcohol XII was transformed into mesylate XIV and bromoacetate XV. From XIII were obtained mesylate XV, bromoacetate XVII, tosylate XVIII, *p*-fluorosulfonylbenzoate XIX, toluate XX, and bromide XXI.



I, $n = 3$; R = O-*t*-Bu
VIII, $n = 2$; R = CH = CH_2
IX, $n = 3$; R = CH = CH_2

(1) This investigation was supported, in part, by research grants (AM-006900) from the National Institute of Arthritis and Metabolic Diseases and (CA10116) from the National Cancer Institute and, in part, by a training grant (5-T1-GM-555) from the Division of Medical Sciences, National Institutes of Health, U. S. Public Health Service.

(2) A. J. Solo and J. O. Gardner, *Steroids*, **11**, 37 (1968).

(3) In our hands, the Grignard additions gave cleanest products when run as described by Marker[4] (that is, without catalysis by copper ion).

(4) R. E. Marker and H. M. Crooks, *J. Amer. Chem. Soc.*, **64**, 1280 (1942).