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Some Aspects of Substrate Specificity in Biological Demethylation at C4 of Steroids

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In order to ascertain some of the structural requirements for substrate activity in the oxidative demethylation at C4 of steroids by rat liver enzymes, several steroids have been synthesized, labeled with tritium, and incubated with rat liver enzyme preparations. These include 4α-formyl-4β-methylcholestan-3β-ol (**4**), 4-methylcholest-3-ene (**14**), 3β,4β-epoxy-4α-methylcholestane (**20**), 3α,4α-epoxy-4β-methylcholestane (**18**), 4α-ethyl-4β-methylcholestan-3β-ol (**21**), and 4β-ethyl-4α-methylcholestan-3β-ol (**22**). Enzymic incubation demethylates **4** with an efficiency consistent with its being an intermediate in the biological demethylation of 4,4-dimethyl sterols, but all of the other substrates are recovered unchanged.

The oxidative demethylation at C4 during the latter stages of steroid biosynthesis was investigated initially by Bloch and co-workers, who showed that the methyl groups were converted to carbon dioxide (*1*) and obtained other evidence consistent with a mechanism involving β-ketoacid decarboxylations as the mode of loss of carbon (*1*, *2*). Gaylor has conducted an extensive series of investigations (*3*) of various aspects of these biological demethylations, using an "enzymic" approach. He has elucidated a considerable amount of biochemical detail by such means as removing cofactors for certain phases of the multistep demethylation process in order to allow accumulation of intermediate species.

Our research in this area (*4–6*) has been based on the observation that 4,4-dimethylcholestan-3β-ol (**1**) is demethylated to cholestan-3β-ol by the same enzyme system that demethylates the natural unsaturated substrate derived from lanosterol (*4*). By use of

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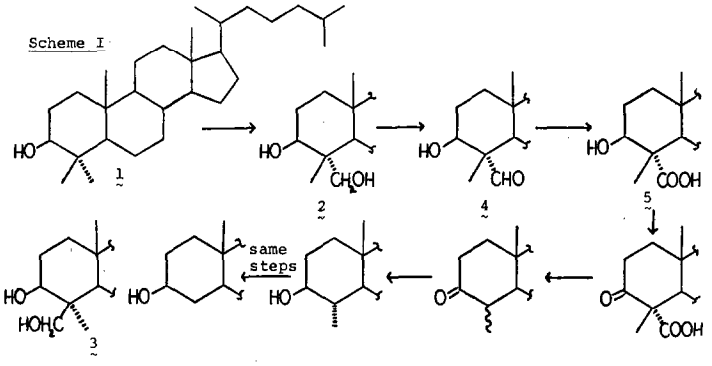
what Gaylor (*3*) terms an "organic" approach, various cholestane derivatives were synthesized, labeled with tritium , and tested as substrates for enzymic demethylation. One of the key findings (*4*) was that, whereas 4α-hydroxymethyl-4β-methylcholestan-3β-ol (**2**) was demethylated more effectively than **1**, 4β-hydroxymethyl-4α-methylcholestan-3β-ol (**3**) was completely unchanged by the same multienzyme rat liver homogenate (*7*). The inference from this result was that the 4α methyl group was the one initially oxidized, in contrast to an earlier claim (*8*) that the 4β methyl was the site of the first enzymic hydroxylation. A double-labeling study (*6*) of the enzymic demethylation of the natural substrate, lanosterol, confirmed that it is indeed the 4α methyl group which is first removed. Evidence was also obtained (*5*) which suggested that the 4β-methyl group was epimerized to the 4α position before it was oxidatively attacked, probably by the same enzyme system. The sequence of conversions shown in Scheme I summarizes all these results. In the present paper are described the synthesis and biological testing of several additional steroids intended to probe the substrate specificity of the enzymic demethylation.



We have reported preliminarily (*5*) that hydroxyacid **5** was converted to cholestan-3β-ol by rat liver homogenate at a rate consistent with its being an intermediate in the demethylation of **1**, and Gaylor has neatly demonstrated (*9*) that the analogous hydroxy acid is an intermediate in the demethylation of lanosterol. To complete the testing of all possible oxidation levels at the 4α substituent we have prepared and incubated 4α-formyl-4β-methylcholestan-3β-ol (**4**).

The syntheses of **4** and **5** from 4α-carbomethoxy-4β-methylcholestan-3β-ol (**8**) (*10*) are outlined in Scheme II, and details are given in the Experimental Section. For comparison, the isomeric hydroxyaldehyde **6** and hydroxyacid **7** were also prepared as shown in Scheme II, although these were not used as enzymic substrates.

The results of incubation of tritium-labeled dihydrolanosterol, **1**, **2**, **4**, and **5** with the same rat liver homogenate are given in Table 1. It is clear that **4** and **5** qualify as intermediates in C4 oxidative demethylation, being converted to cholestan-3β-ol at least as efficiently as diol **2**. No attempt was made in these experiments to isolate or identify other radioactive products which may have been present.

These results are certainly consistent with β-ketoacid decarboxylation as the mechnism of loss of the C4 methyl carbons. Gaylor's demonstration that the natural intermediate analogous to **5** accumulates when oxidized pyridine nucleotide is excluded

Scheme II

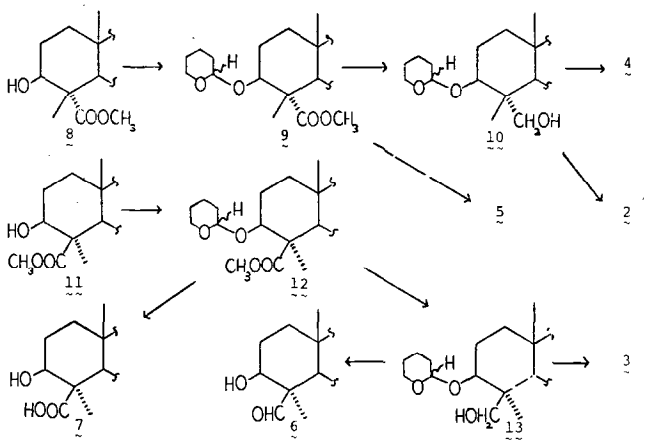


TABLE 1

RESULTS OF INCUBATION OF SEVERAL STEROIDS WITH RAT LIVER HOMOGENATE

Substrate	Conversion to cholestan-3β-ol[a] (%)	Recovered substrate[a] (%)
Dihydrolanosterol	82[b]	9[b]
4,4-Dimethylcholestan-3β-ol (**1**)	14[c]	78[c]
4α-Hydroxymethyl-4β-methylcholestan-3β-ol (**2**)	17[c,d]	63[c]
4α-Formyl-4β-methylcholestan-3β-ol (**4**)	24[c]	51[c,e]
4α-Carboxyl-4β-methylcholestan-3β-ol (**5**)	17[c]	47[c,e]

[a] Values refer to percentage of total recovered radioactivity, which was typically 70–80% of starting radioactivity. See Experimental Section and references therein for details.

[b] Average of three incubations; product was cholesterol.

[c] Average of two incubations.

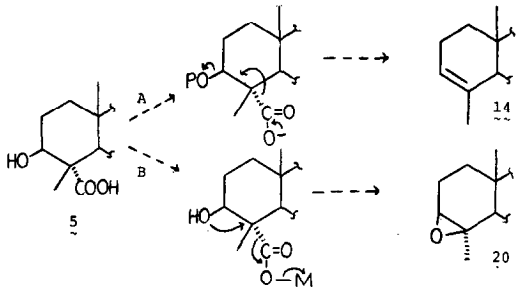
[d] With an earlier enzyme preparation, **2** was much more effectively demethylated than **1** (*4*).

[e] Based on recovered **2**; see Experimental Section.

from the enzyme preparation (*9*) is even more suggestive of β-ketoacid decarboxylation. However, there are other *possible* mechanisms for the loss of carbon dioxide, and two of these are shown in Scheme III. In IIIA a decarboxylative elimination (*11*) to 4-methylcholest-3-ene (**14**) is proposed, analogous to that in the conversion of phosphorylated mevalonic acid to isopentenyl pyrophosphate (*12*). This pathway requires presumably irreversible loss of the C3 oxygen, but this possibility could not be ruled out since it has not been established (*13*) whether the oxygen atom originally incorporated is retained beyond the lanosterol stage in steroid biosynthesis. Subsequent reintroduction of oxygen at C3 (as, for example, by conversion to **20**) would be required for the second demethylation (*3*, *5*).

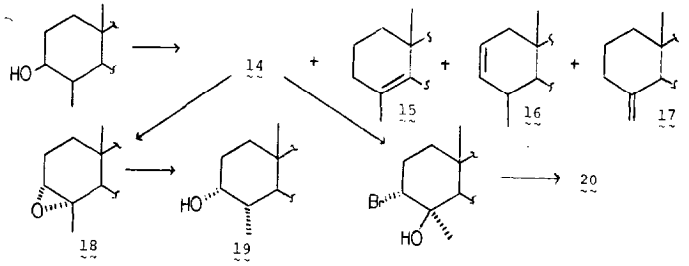
Scheme IIIB postulates an oxidative decarboxylation in which an incipient or actual carbonium ion at C4 is trapped by the C3 hydroxyl acting as a nucleophile to form

Scheme III



oxide **20**. Oxidative decarboxylation of structurally similar acids with, for example, lead tetraacetate, is well documented (*14*), although we are unaware of an example of ensuing epoxide formation by a neighboring oxygen.

Olefin **14** was separated by chromatography on $AgNO_3$-impregnated florisil from the mixture of alkenes **14–17** obtained by treatment of 4β-methylcholestan-3β-ol (*10*, *15*) with $POCl_3$ in pyridine. 4β-Methylcholestan-3β-ol was used because tlc analysis showed that it afforded a considerably larger fraction of the desired Δ^3 compound upon dehydration than its more readily available 4α-methyl isomer. Peracid epoxidation of cholest-3-ene leads to 3α,4α-epoxycholestane (*16*), so when only a single oxide was obtained upon treatment of **14** with *m*-chloroperbenzoic acid it was thought to be the α-oxide **18**. This was shown to be the case by conversion of the compound with $LiAlH_4$ to the known (*17*) 4α-methylcholestan-3α-ol (**19**). The desired β-oxide **20** was obtained by an alternate route involving treatment of **14** with aqueous *N*-bromosuccinimide to form an intermediate bromohydrin, which was converted to **20** by treatment with K_2CO_3 in methanol.

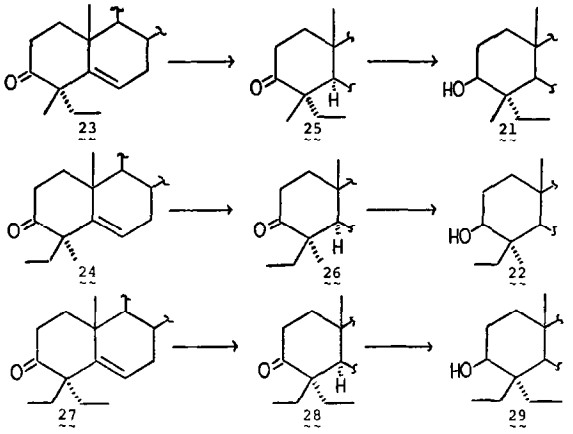


Tritium-labelled **14**, **18**, and **20** were each subjected to the usual incubation with active rat liver homogenate. In each case, a radiochromatogram scanner trace of the product was identical with that of the product from incubation of substrate with a boiled (inactive) enzyme preparation. The fact that **14**, **18**, and **20** were recovered unchanged is evidence against the proposals represented in Scheme III.

Finally, we have been attempting to gain some insight into the steric requirements of a substrate for effective hydroxylation at a C4 substituent by rat liver enzymes. The apparently complete lack of reactivity of the 4β-hydroxymethyl compound **3** is striking; the replacement of H by OH at the C4 β-methyl (**1** → **3**) produces a substance that is not detectably demethylated.

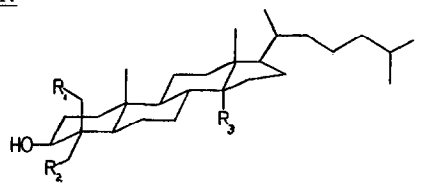
To seek to determine whether this was an electronic or a steric effect, the isomeric 4-ethyl-4-methyl sterols **21** and **22** were selected as substrates. An analogous homolog of 2,3-oxidosqualene bearing an ethyl and a methyl rather than two methyls on the oxirane ring has been found (*18*) to be fairly efficiently enzymically converted to a homolanosterol. If a comparable indifference to homologation were found for demethylation of **22**, the unacceptability of **3** as a substrate would clearly not be steric in origin.

Synthesis of **21** and **22** was readily accomplished by hydrogenation of the known (*19*) **23** and **24** to produce **25** and **26**, which were reduced with $NaBH_4$ to **21** and **22**, respectively. Since the diethyl compound **27** was obtained as a byproduct during the preparation of **24** (*19*), it was converted in the same manner to **28** and **29**, in order that the latter could be used as a substrate if **21** and **22** were enzymically oxidized.



Neither **21** nor **22**, however, was detectably affected by an active enzyme preparation, with well over 99% of the recovered radioactivity being found in the recovered starting material. Clearly, the active site of the hydroxylating enzyme in the initial step of the demethylation sequence cannot accommodate much change in size near C4 in potential substrates, and the failure of **3** to be metabolized may well be due to steric factors. Studies of the effects of other modifications in the region of C4 are underway, as are

Scheme IV



	Substrate	C4 demethylation	Reference
1	$R_1 = R_2 = R_3 = H$	Yes	*4*
2	$R_1 = R_3 = H$; $R_2 = OH$	Yes	*4*
3	$R_1 = OH$; $R_2 = R_3 = H$	No	*4*
30	$R_1 = R_2 = H$; $R_3 = CH_3$	No	*4*
21	$R_1 = R_3 = H$; $R_2 = CH_3$	No	This paper
23	$R_1 = CH_3$; $R_2 = R_3 = H$	No	This paper

studies of structural changes in other portions of the steroid. In connection with the latter studies, it should be noted that we have already observed (*4*) that the 14α methyl group in **30** inhibits enzymic attack on the C4 methyl groups. Scheme IV summarizes our results to date, which indicate a significant substrate specificity in the saturated model series that presumably (*4*) obtains in the natural series as well.

EXPERIMENTAL SECTION

Synthesis

Melting points were determined in open capillaries in a Thomas–Hoover apparatus and are uncorrected. Unless otherwise specified, ir spectra of solids were obtained as KBr pellets and liquids as neat films on a Perkin–Elmer 137 spectrophotometer. Nmr spectra were determined on a Perkin–Elmer R-24 spectrometer with TMS as an internal standard. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Preparative tlc (prep tlc) was performed on 20 × 20-cm plates coated with 1.45-mm-thick layers of silica gel PF (Brinkman Instruments Inc., Westbury, N.Y.). Ultraviolet light was used to visualize tlc plates. Brine refers to saturated aqueous sodium chloride solution.

4α-Carbomethoxy-4β-methylcholestan-3β-ol (**8**) *and 4β-Carbomethoxy-4α-methylcholestan-3β-ol* (**11**)

Hydroxyesters **8** and **11** were prepared by reductive carbomethoxylation of Δ^4-cholesten-3-one, methylation of the resulting 4α-carbomethoxycholestan-3-one to give both stereoisomeric methylated β-keto esters, and $NaBH_4$ reduction. Details are given in Ref. (*10*).

3β-[(Tetrahydro-2H-pyran-2-yl)oxy]-4α-carbomethoxy-4β-methylcholestane (**9**)

A solution containing 1.2 g (2.6 mmol) of **8** and 1.0 g (12.0 mmol) of 2,3-dihydropyran (bp 85–86°C) in 30 ml of dry methylene chloride was treated with dry hydrogen chloride for 5 min at 0°C. The mixture was stored at room temperature for 72 hr, poured into 50 ml of 1 *M* $NaHCO_3$, and extracted with ether. The organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 1.4 g of a yellow viscous oil. Analysis of this oil by tlc (1:1, ether:hexane, twice) showed two substances with R_f = 0.60 and 0.66 (R_f of (**8**) = 0.10), presumably the two diastereomeric tetrahydropyranyl ethers. Crystallization of the oil from methanol (with difficulty) gave 1.2 g (84%) of the diastereomeric mixture **9**, mp 99–105°C; ir 1740 cm^{-1}; nmr ($CDCl_3$) δ 0.65 (s, C18), 0.90 (s, C19), 1.20 (s, 4β H_3C–), 3.65 (s, H_3COOC–), 3.4–4.2 (bm), 4.5 (bs), and 4.8 ppm (bs).

Anal. Calcd for $C_{35}H_{60}O_4$: C, 77.15; H, 11.10. Found: C, 77.18; H, 11.05.

3β-[(Tetrahydro-2H-pyran-2-yl)oxy]-4α-hydroxymethyl-4β-methylcholestane (**10**)

To a stirred solution of 0.196 g (0.36) mmol) of **9** in 25 ml of anhydrous ether at room temperature was added 0.050 g (1.3 mmol) of $LiAlH_4$. After 6 hr, the excess $LiAlH_4$ was destroyed by the addition of ethyl acetate, followed by 50 ml of 5% KOH solution.

The mixture was extracted with ether, and the organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.172 g of crystalline material, mp 115–135°C. Analysis of this material by tlc (1:1, ether:hexane, twice) showed two substances with $R_f = 0.26$ and 0.32, plus traces of **9** and **2** ($R_f = 0.13$). The material was purified by prep tlc (1.25:1, ether:hexane) to give 0.151 g (81%) of the diastereomeric mixture **10**. Recrystallization from methanol–ether gave 0.092 g (50%) of **10**, mp 143–146°C; ir 3500 cm^{-1}; nmr ($CDCl_3$) δ 0.65 (s, C18), 0.90 (s, C19 and 4β H_3C–) and 3.35–4.7 ppm (bm).

Anal. Calcd for $C_{34}H_{60}O_3$: C, 79.01; H, 11.70. Found: C, 79.08; H, 11.78.

In large scale preparations, column chromatography (florisil, Grade III) was used to isolate **10**. Elution with ether:hexane, 1:9, gave one crystalline diastereomer of **10** ($R_f = 0.32$) mp 152–158°C; ir (KBr) 3575 cm^{-1}; elution with ether:hexane, 1:2, gave the other diastereomer ($R_f = 0.26$), mp 118–128°C; ir 3400 cm^{-1}.

Treatment of a small sample of each of the diastereomers with 10% HCl in ethanol gave diol **2** as the only product by tlc analysis.

4α-Formyl-4β-methylcholestan-3β-ol (**4**)

To a stirred solution containing 0.083 g (0.18 mmol) of **10** in 50 ml of acetone at 0°C was added 5 drops of Jones' reagent (*20*). After 20 min, 0.5 g of anhydrous K_2CO_3 was added, and the mixture was filtered. The acetone solution was concentrated *in vacuo* to give 0.095 g of the crude tetrahydropyranyl aldehyde, ir (neat) 1710 and 2700 (weak) cm^{-1}. A solution of 0.080 g (0.155 mmol) of this material in 15 ml of ethanol was treated with 10 drops of 10% HCl and refluxed for 15 min. The cooled solution was concentrated, 50 ml of water was added, and the mixture was extracted with ether. The organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give a colorless, viscous oil. Crystallization from ethanol–ether gave 0.040 g (52%) of **4**, mp 161–164°C (dec). Recrystallization from ethanol gave an analytical sample, mp 162–164°C (dec); ir 3410, 2700 (weak), and 1705 cm^{-1}; nmr ($CDCl_3$) δ 0.64 (s, C18), 0.90 (s, C19), 1.07 (s, 4β H_3C–), 3.5–4.0 (bm, 3α H), and 9.64 ppm (s, H—C$\overset{O}{<}$).

Anal. Calcd for $C_{29}H_{50}O_2$: C, 80.87; H, 11.70. Found: C, 80.65; H, 11.59.

3β-Hydroxy-4β-methylcholestan-4α-carboxylic acid (**5**)

A solution containing 0.60 g (1.1 mmol) of **9**, 1.0 g (18.0 mmol) of KOH, and 55 ml of water:isopropanol, 1:9, was refluxed under nitrogen for 48 hr. The cooled mixture was poured into 100 ml of water, acidified with 10% HCl, and refluxed for 10 min. The cooled mixture was made basic to pH ~12 with 5% NaOH solution. The precipitate which formed was removed by filtration, slurried in ether, and filtered. This filtrate was concentrated to give 0.115 g of semisolid neutral material. Analysis by tlc (1.5:1, ether:hexane) indicated several components including principally **8**.

The remaining solid, presumably the sodium salt of **6**, was treated with 10% HCl and extracted with ether. The organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.330 g (67%) of **5**, mp 260–270°C (dec). Recrystallization from methanol–ether gave pure **5**, mp 275–277°C (dec); ir 3450, 3225, and 1680 cm^{-1}.

Anal. Calcd for $C_{29}H_{50}O_3$: C, 77.97; H, 11.28. Found: C, 77.84; H, 11.18.

3β-[(Tetrahydro-2H-pyran-2-yl)oxyl]-4β-carbomethoxy-4α-methylcholestane (**12**)

Exactly as in the preparation of **9** from **8**, 0.190 g (0.41 mmol) of **11** was converted to 0.178 g (80%) of a mixture of diastereomers of **12**, mp 86–92°C. No tlc separation of the diastereomers was observed. Recrystallization from methanol–ether gave an analytical sample, mp 104–109°C; ir 1725 and 1730 cm^{-1}; nmr ($CDCl_3$) δ 0.64 (s, C18), 0.80 (s, C19), 1.23 and 1.35 (4β-H_3C's), 3.60 and 3.62 (H_3COOC's) and 3.2–4.8 ppm (bm).

Anal. Calcd for $C_{35}H_{60}O_4$: C, 77.15; H, 11.10. Found: C, 77.02; H, 11.13.

3β-[(Tetrahydro-2H-pyran-2-yl)oxy]-4β-hydroxymethyl-4α-methylcholestane (**13**)

Exactly as in the preparation of **10** from **9**, 0.070 g (0.13 mmol) of **12** gave 0.064 g (95%) of a mixture of the diastereomers of **13**, mp 133–140°C. No tlc separation was observed. Recrystallization from methanol–ether gave an analytical sample, mp 144–148°C; ir 3600 cm^{-1}; nmr ($CDCl_3$) δ 0.62 (s, C18), 0.81 (s), 0.91 (s), and 3.2–4.8 ppm (bm).

Anal. Calcd for $C_{34}H_{60}O_3$: C, 79.01; H, 11.70. Found: C, 78.92; H, 11.68.

Treatment of a small sample of **13** with 10% HCl–ethanol gave only **3** by tlc analysis.

4β-Formyl-4α-methylcholestan-3β-ol (**6**)

Exactly as in the preparation of **4** from **10**, 0.176 g (0.34 mmol) of **13** gave 0.140 g (94%) of crude **6**, mp 142–150°C (dec). Recrystallization from ethanol afforded pure **6** as white needles, mp 160–166°C (dec); ir 3580 and 1695 cm^{-1}; nmr ($CDCl_3$) δ 0.65 (s, C18), 0.80 (s, C19), 1.26 (s, 4α H_3C–), 3.0–3.6 (bm, 3α H), and 9.8 ppm (m, $HC(=O)$—).

Anal. Calcd for $C_{29}H_{50}O_2$: C, 80.87; H, 11.70. Found: C, 80.92; H, 11.73.

3β-Hydroxy-4α-methylcholestan-4β-carboxylic acid (**7**)

According to a reductive hydrolysis procedure reported by Wenkert (*21*), a solution of 0.200 g (0.28 mmol) of **12** in 25 ml of dry tetrahydrofuran and 75 ml of liquid ammonia was treated with 10 mg (1.8 mmol) of lithium wire with vigorous stirring. After 1 hr, the mixture was warmed to room temperature, and the ammonia was allowed to evaporate overnight. A few drops of ethanol were added to destroy the excess lithium. Water (50 ml) was added, and the mixture was extracted with ether. The ether layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.024 g of a gum, ir (neat) 3585 cm^{-1}. Tlc (1:1 ether:hexane) indicated one major product which had the R_f of **13**.

The original aqueous layer was acidified with 10% HCl and extracted with ether. The organic layer was washed with brine, dried ($MgSO_4$), and concentrated to give 0.153 g of the tetrahydropyranyl ether of **7**, mp 182–188°C; ir (KBr) 3400–3200 (broad) and 1710 cm^{-1}. Without purification, 0.070 g (0.13 mmol) of this material in 10 ml of ethanol was treated with 1 ml of 10% HCl and refluxed for 20 min. The standard ether workup afforded 0.050 g (85%) of **7**, mp 220–230°C (dec). Recrystallization from ethanol gave pure **7**, mp 235–237°C (dec): ir 3400, 2600, and 1675 cm^{-1}.

Anal. Calcd for $C_{29}H_{50}O_3$: C, 77.97; H, 11.28. Found: C, 78.08; H, 11.39.

Dehydration of 4β-Methylcholestan-3β-ol

A solution of 0.308 g (0.75 mmol) of 4β-methylcholestan-3β-ol (*10*, *15*), and 0.84 g (0.5 ml; 5.5 mmol) of $POCl_3$ in 4 ml of dry pyridine was heated on a steam bath for 20 min. The mixture was kept at room temperature for 3.5 hr and concentrated *in vacuo*. Ether was added to the residue and the resulting slurry was filtered through 15 g of florisil. The filtrate was evaporated to give 0.299 g of a colorless semisolid product that tlc on 12.5% $AgNO_3$–silica gel PF (*22*), using ether:hexane, 1:99, twice, showed to contain four substances with R_f's = 0.25, 0.53, 0.75, and 0.80.

For isolation of 4-methylcholest-3-ene (**14**) several such products were combined and column chromatographed. Typically, 1.62 g of product from dehydration of 1.77 g of 4β-methylcholestan-3β-ol was chromatographed on 129 g of 19% $AgNO_3$–florisil (*22*). Elution with hexane gave 0.218 g (13%) of 4-methylcholest-4-ene (**15**) as a colorless oil (R_f = 0.80). Crystallization from ether–ethanol (with difficulty) gave 0.135 g of **15** mp 59–61°C (lit. (*23*) mp 56–58°C and lit. (*24*) mp 52–53°C); nmr (CCl_4) δ 0.64 (s, C18), 0.80 (s, C19), and 1.53 ppm (s, $H_3C-\underset{|}{C}=C$). Elution with ether:hexane, 1:99, gave 0.809 g (48%) of solid 4-methylcholest-3-ene (**14**) (R_f = 0.75). Recrystallization from ether–ethanol gave 0.546 g of **14**, mp 82–85°C (lit. (*17*) mp 85–87°C); ir (CS_2) 3027 (w), 1660 (vw) and 795 cm^{-1} (w); nmr (CCl_4) δ 0.64 (s, C18), 0.71 (s, C19), 1.54 (s, $H_3C-\underset{|}{C}=C$), and 5.17 ppm (m, 1, $H-\underset{|}{C}=C$); M^+ (*m/e*) 384.

Anal. Calcd for $C_{28}H_{48}$: C, 87.42; H, 12.58. Found: C, 87.51; H, 12.64.

Elution with ether:hexane, 7:93, gave 0.202 g (12%) of 4β-methylcholest-2-ene (**16**) as a colorless oil (R_f = 0.53). Crystallization from ether–ethanol gave 0.138 g of **16**, mp 81–84°C; ir (CS_2) 3000, 1650 (w), and 728 cm^{-1} (s); nmr (CCl_4) δ 0.62 (s, C18), and 5.42 ppm (m, 2, (*H*—C=C—H); M^+ (*m/e*) 384.37560 (calcd for $C_{28}H_{48}$: 384.37558).

Elution with ether:hexane, 3:7, gave 0.198 g (12%) of 4-methylenecholestane (**17**) as a colorless oil (R_f = 0.25). Crystallization from ether:ethanol gave 0.127 g of **17**, mp 83–86°C (lit. (*25*) mp 84–86°C); ir (CS_2) 3075 (w), 1695 (m), and 887 cm^{-1} (s); nmr (CCl_4) δ 0.66 (s, C18), 0.69 (s, C19), and 4.48 ppm (*d*, 2, *J* = 15 Hz, $H_2C=C$).

3α,4α-Epoxy-4β-methylcholestane (**18**)

To a stirred solution of 0.300 g (0.78 mmol) of **14** in 30 ml of methylene chloride at 0°C was added dropwise a solution of 0.182 g (0.90 mmol) of 85% *m*-chloroperbenzoic acid in 20 ml of methylene chloride. The mixture was stirred at room temperature for 1 hr, poured into 100 ml of 1 *M* $NHCO_3$ solution, and extracted with ether. The organic layer was washed with 1 *M* $NaHCO_3$ solution and brine, dried ($MgSO_4$) and concentrated to give 0.352 g of white semisolid material. Purification by prep tlc (1:4, ether:hexane) gave 0.248 g (79%) of **18**, mp 94–96°C. Recrystallization from ethanol–ether gave an analytical sample, mp 96–97°C; ir 1260 and 800 cm^{-1}; nmr ($CDCl_3$) δ 0.65 (s, C18), 0.75 (s, C19), 1.25 (s, 4β-H_3C–) and 2.98 ppm (bs, 3β *H*); M^+ (*m/e*) 400.

Anal. Calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 84.22; H, 12.22.

Lithium Aluminium Hydride Reduction of (**18**)

A stirred suspension of 0.150 g (0.37 mmol) of **18** and 0.300 g (7.9 mmol) of $LiAlH_4$ in 20 ml of tetrahydrofuran, which has been distilled from $LiAlH_4$, was refluxed for

56 hr. The mixture was cooled and excess $LiAlH_4$ was destroyed by slow addition of water, followed by the addition of 10% HCl solution. The aqueous solution was extracted with ether and the organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.172 g of yellow semisolid material, which tlc (3:7 ether:hexane) indicated was a mixture of three compounds.

Separation was effected by prep tlc using ether:hexane, 3:7. There were obtained 0.009 g of **18** ($R_f = 0.45$) and 0.079 g (53%) of 4α-methylcholestan-3α-ol (**19**) ($R_f = 0.21$). Recrystallization of **19** from ethanol gave 0.056 g, mp 114–115°C (lit. (*17*) mp 116–117°C); ir 3450 cm^{-1}; nmr (CCl_4) δ 0.63 (s, C18), and 3.63 ppm (bs, 3β *H*). Compound **19** was treated with acetic anhydride in pyridine at room temperature for 72 hr. The usual workup and recrystallization from ethanol afforded the acetate derivative as white needles, mp 112–113°C (lit. (*17*) mp 112–113°C); ir 1735 and 1240 cm^{-1} (lit. (*17*) 1740 and 1240 cm^{-1}); nmr (CCl_4) δ 0.65 (s, C18), 1.98 (s, H_3C—C(=O)—), and 4.82 ppm (bs, 3β *H*).

The third compound (0.053 g; $R_f = 0.13$) was recrystallized from methanol:ether to mp 190–193°C; ir (CS_2) 3580 cm^{-1}; nmr (CCl_4) δ 0.65 ppm (s, C18) and no *H*—C(—)—OH. This material, presumably 4β-methycholestan-4α-ol, was not characterized further.

3β,4β-Epoxy-4α-methylcholestane (**20**)

A mixture of 0.150 g (0.39 mmol) of **14**, 0.150 g (0.84 mmol) of *N*-bromosuccinimide, 5 ml of water and 50 ml of dimethoxyethane was stirred at room temperature for 3 hr. Two milliliters of 5% K_2CO_3 solution was added and the mixture was concentrated *in vacuo*. The residue was purified by prep tlc (1:7, ether:hexane) to give 0.080 g of presumed bromohydrin, ir (neat) 3400 cm^{-1}. A mixture of this material, 2 ml of 5% K_2CO_3 solution, and 20 ml of methanol was refluxed for 3 days, and the mixture was concentrated *in vacuo*. The residue was partitioned between water and ether. The organic layer was washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.073 g of crude **20**. Purification by prep tlc (1:7, ether:hexane) and recrystallization from ethanol–ether gave 0.022 g (14%) of pure **20**, mp 113–114°C; ir 850 cm^{-1}; nmr ($CDCl_3$) δ 0.65 (s, C18), 0.90 (s, C19), 1.22 (s, 4α H_3C–), and 2.88 ppm (bm, 3α *H*); M^+ (*m/e*) 400.

Anal. Calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 84.21; H, 12.23.

4α-Ethyl-4β-methylcholestan-3-one (**25**).

An improved procedure for the hydrogenation of 4,4-dimethylcholest-5-en-3-one (**31**) (*26*) to 4,4-dimethylcholestan-3-one (**32**) was worked out and applied to the preparation of **25**. Using this procedure, described in detail below, hydrogenation of **31** afforded a crude product which glc analysis showed to be a 1:1:18 mixture of **1**, 4,4-dimethylcoprostan-3-one (*27*) and **32**. After treatment with Jones reagent (*20*), 96% of **32**, mp 94–97°C, was obtained. Recrystallization from ethanol gave 80% of **32**, mp 100–101°C (lit. (*28*) mp 100–101°C), >99% pure by glc.

For preparation of **25**, a stirred mixture of 0.190 g (0.45 mmol) of 4α-ethyl-4β-methylcholest-5-en-3-one (**23**), prepared by the method of Just and Richardson (*19*), 0.08 g of 10% palladium-on-carbon, 20 ml of cyclohexane, and 20 ml of glacial acetic acid was hydrogenated for 3 hr at room temperature and atmospheric pressure. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*, using added benzene to remove the acetic acid by azeotropic distillation. The crude product was purified by prep tlc, using ether:hexane, 1:4, three times, to give solid **25**, which was recrystallized from ethanol to afford 0.151 g (79%) of **25**, mp 101–103°C; ir 1710 cm^{-1}; nmr (PhH) δ 0.67 (s, C18), 0.77 (s, C19), and 0.97 ppm (s, 4β H_3C–).

Anal. Calcd for $C_{30}H_{53}O$: C, 84.04; H, 12.22. Found: C, 83.93; H, 12.08.

4α-Ethyl-4β-methylcholestan-3β-ol (**21**)

A mixture of 0.120 g (0.28 mmol) of **25**, 0.060 g (1.57 mmol) of $NaBH_4$, and 50 ml of methanol was stirred for 3 hr at room temperature. The methanol was evaporated *in vacuo*, and the resulting solid was partitioned between 50 ml of ether and 25 ml of 10% HCl. The ether layer was separated, dried ($MgSO_4$), and concentrated to give 0.120 g of solid which was purified by prep tlc, using ether:hexane, 1:3, to afford 0.078 g of crude **21**. Recrystallization from methanol gave 0.070 g (60%) of pure **21**, mp 134–135°C; ir 3500 cm^{-1}; nmr (PhH) δ 0.68 (s, C18), 0.85 (s), 0.95 (s), and 3.28 ppm (bt, 3α *H*).

Anal. Calcd for $C_{30}H_{54}O$: C, 83.65; H, 12.64. Found: C, 83.62; H, 12.79.

4β-Ethyl-4α-methylcholest-5-en-3-one (**24**)

To a mixture of 50 ml of dry *t*-butyl alcohol and 0.337 g (8.3 mmol) of NaH (57% dispersion in mineral oil) under nitrogen, was added 0.840 g (2.04 mmol) of 4-ethylcholest-4-en-3-one (*19*). The resulting mixture was refluxed for 1 hr and then a solution of 1.0 g (7.0 mmol) of methyl iodide in 10 ml of dry *t*-butyl alcohol was added dropwise over 30 min. After being refluxed for 12 hr, the mixture was cooled and evaporated *in vacuo*. The resulting yellow gum was dissolved in 100 ml of ether and washed with 5% HCl solution, water and brine. The organic layer was dried ($MgSO_4$) and concentrated to give 1.1 g of a yellow semisolid material, which was chromatographed on 20 g of florisil (Grade I) in hexane. Elution with ether:hexane, 1:12, gave 0.394 g (45%) of **24**, mp 125–130°C. Recrystallization from ethanol–ether afforded 0.344 g (40%), mp 151–153°C (lit. (*19*) mp 153.5–154°C); ir 1705 cm^{-1} (lit. (*19*) ir (CCl_4) 1710 cm^{-1}); nmr (PhH) δ 0.67 (s, C18), 0.75 (s, C19) and 1.14 ppm (s, 4α H_3C–).

Further elution with ether gave 0.416 g of 4-ethylcholest-4-en-3-one.

4β-Ethyl-4α-methylcholestan-3-one (**26**)

Exactly as in the preparation of **25** from **23**, 0.250 g (0.59 mmol) of **24** gave 0.204 g (81%) of crude **26**. Crystallization from ethanol afforded 0.100 g (40%) of pure **26** (*29*), mp 102–104°C; ir 1700 cm^{-1}; nmr (PhH) δ 0.67 (s, C18), 0.91 (s, C19) and 1.13 ppm (s, 4α H_3C–).

Anal. Calcd for $C_{30}H_{52}O$: C, 84.04; H, 12.22. Found: C, 84.03; H, 12.19.

4β-Ethyl-4α-methylcholestan-3β-ol (**22**)

Exactly as in the preparation of **21** from **25**, 0.085 g (0.20 mmol) of **26** gave 0.055 g (64%) of crystalline **22**, mp 124–127°C; ir 3500 cm^{-1}; nmr (PhH) δ 0.68 (s, C18), 0.82 (s, C19), 1.02 (s, C4α H_3C–) and 2.8–3.2 ppm (bm, 3α *H*).

Anal. Calcd for $C_{30}H_{54}O$: C, 83.65; H, 12.64. Found: C, 83.82; H, 12.51.

4,4-Diethylcholestan-3-one (**28**)

Exactly as in the preparation of **25** from **23**, 0.076 g (0.17 mmol) of 4,4-diethylcholest-5-en-3-one (**27**) (*19*) gave 0.060 g (80%) of crystalline **28** (*29*), mp 109–110°C; ir 1705 cm^{-1}; nmr (PhH) δ 0.67 (s, C18), and 0.93 ppm (s, C19); M^+ (*m/e*) 442.41726 (calcd for $C_{31}H_{54}O$: 442.41745).

4,4-Diethylcholestan-3β-ol (**29**)

Exactly as in the preparation of **21** from **25**, 0.070 g (0.16 mmol) of **28** gave 0.043 g (62%) of **29**, mp 118–119°C; ir 3400 cm^{-1}; nmr ($CDCl_3$) δ 0.64 (s, C18) and 3.4–3.65 ppm (bt, 3α *H*); M^+ (*m/e*) 444.43297 (calcd for $C_{31}H_{56}O$: 444.43310).

Isotopic Labelling

All substances used in incubations with rat liver enzymes were labeled with ^3H by exchange of protons α to carbonyl groups exactly according to the procedure described by Nadeau and Hanzlik (*30*). The compound so labeled for each (substrate) was: 4α-carbomethoxy-4β-methylcholestan-3-one (*10*) (**4**, **5**); 4-methylcholest-4-en-3-one (*31*) (**14**, **18**, **20**); **25** (**21**); **26** (**22**).

Enzyme Preparations

Bucher–McGarrahan rat liver homogenates were prepared according to the procedure described by Popjak (*32*) and used as described therein with labeled **4**, **5**, **14**, **18**, and **20**. The modified procedure of Gaylor (*9*) was used to prepare the enzyme mixture employed in the incubations of **21** and **22**.

Incubations

The incubations were run as previously described (*6*). At the end of the incubation period, work-up was conducted as follows. For **4** and **5**, the incubation mixture was extracted with ether, and the ether extracts were treated for 1 hr with excess $LiAlH_4$. For **14**, **18**, and **20**, the incubation mixture was diluted with 5 ml of 20% KOH in ethanol and heated at 50°C overnight. For **21** and **22**, the incubation mixture was diluted with acetone and then filtered to remove precipitated protein.

Each of the product mixtures described above was partitioned between ether and water, and the residue from the ether layer was analyzed by tlc exactly as previously described (*6*), using a Packard Model 7201 radiochromatogram scanner. Bands were separated, eluted, and counted for ^3H on a Packard Model 3320 TR1-CARB liquid

scintillation counter. In the case of **4** and **5**, the amount of diol **2** obtained, via the $LiAlH_4$ workup procedure described above, was taken as the measure of unreacted **4** or **5**. Products were identified by comparison of tlc behavior with that of authentic samples; confirmation by comparison of glc retention times was obtained in several cases. Glc was performed on a Varian Model 2100 instrument using 3% QF-1 packing in a 6 ft × 4-mm glass column at 240°C.

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