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Preparation of cholesterol-25-^{3}H

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SUMMARY

*The synthesis of cholesterol-25-^{3}H (**9**) described here starts with the readily available 3β-hydroxy-Δ^5-cholenic acid (**1**). The acetate of this acid was converted to the next higher homolog by the Arndt-Eistert method. Grignard reaction with methyl iodide gave 25-hydroxycholesterol (**6**), which was then brominated. Tritium was finally introduced by treating the 25-bromocholesterol (**8**) with $LiAl^3H_4$.*

INTRODUCTION.

It is generally assumed that the last step in the biosynthesis of cholesterol involves the reduction of desmosterol (Δ^{24}-dehydrocholesterol) [1]. However, recent experiments with plants have raised the question whether or not this step is reversible [2, 3]. This question can best be answered by the administration of cholesterol, tritiated at either C-24, C-25, or both. We have elected the synthesis of cholesterol-25-^{3}H from 3β-hydroxy-Δ^5-cholenic acid, a commercially available oxidation product of cholesterol.

The steps in the synthesis are outlined in Figure 1 below. New products were characterized by the usual methods. The radiochemical purity of cholesterol-25-^{3}H (**9**) was established by dilution with authentic cholesterol and recrystallization to constant specific activity (Table I). Finally, proof was

TABLE I. Recrystallization of Cholesterol-25-^{3}H

Material	m.p.	Radioactivity in dpm × 10^3/mmole
Diluted with carrier (1 : 430)		12,640
After 1st crystallization	147 -148 °C	12,900
After 2nd crystallization	147.5-148.5 °C	12,780

obtained that no tritium had entered the steroid nucleus by degrading cholesterol-25-^{3}H (**9**) to Δ^5-androsten-3β-ol-17-one. This compound, isolated as the semicarbazone acetate (**10**), showed no radioactivity.

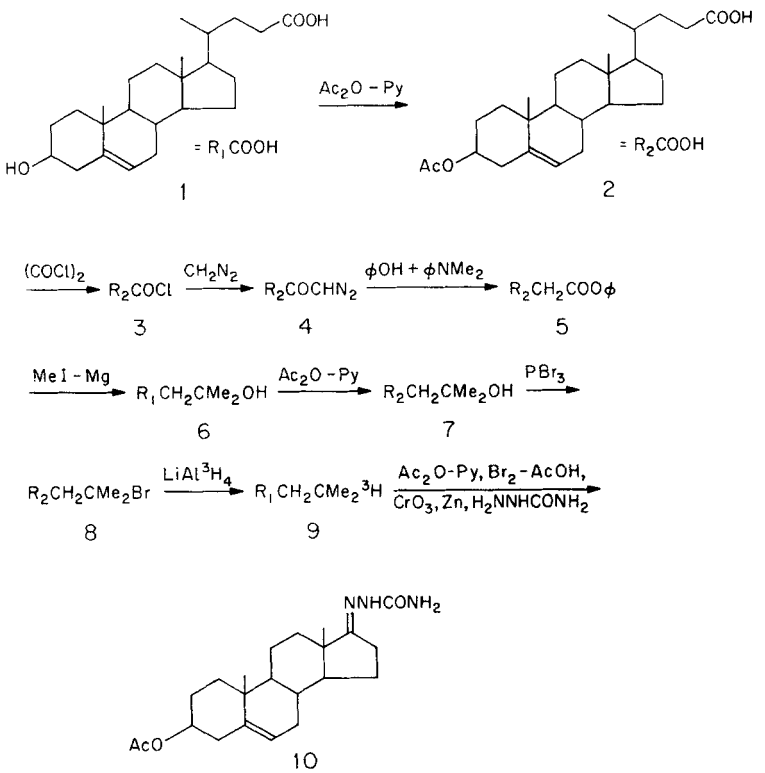

FIG. 1.

EXPERIMENTAL.

*3β-Hydroxy-Δ^5-cholenic acid acetate (**2**) from 3β-hydroxy-Δ^5-cholenic acid (**1**).*

One hundred mg (0.267 mmole) 3β-hydroxy-Δ^5-cholenic acid (**1**) * was acetylated with 0.4 ml pyridine and 0.3 ml acetic anhydride at 23 °C for 20 hrs. Then 0.3 ml water was added and the mixture was refluxed for 1 hr. The work-up consisted in dissolving the product in dichloromethane or ether, washing the solution with water, 2*N* Na_2CO_3, 1*N* HCl, and water in succession, and finally evaporating it to dryness *in vacuo*. This gave 103.5 mg (95.5 %) of 3β-hydroxy-Δ^5-cholenic acid acetate (**2**). After recrystallization from dichloro-

* Purchased from K. and K. Laboratories, Inc., Hollywood, California.

methane-acetone, pure 3β-hydroxy-Δ^5-cholenic acid acetate (**2**) was obtained as shiny leaflets, m.p. 180-181 °C *, lit. m.p. 183-185 °C [4].

*26,27-Bisnor-25-diazo-Δ^5-cholesten-3β-ol-24-one acetate (**4**) from 3β-hydroxy-Δ^5-cholenic acid acetate (**2**).*

One hundred mg (0.240 mmole) 3β-hydroxy-Δ^5-cholenic acid acetate (**2**) was dissolved in 4 ml benzene, and a cooled solution of 0.4 ml oxalyl chloride in 1 ml benzene was added. After 1 1/2 hr, the solution was evaporated to dryness *in vacuo*, and the residue was dried overnight over potassium hydroxide. Yield : 102.2 mg (98.0 %) of colorless, crystalline 3β-hydroxy-Δ^5-cholenic acid chloride acetate (**3**).

Fifty mg (0.115 mmole) 3β-hydroxy-Δ^5-cholenic acid chloride acetate (**3**) was dissolved in 0.5 ml absolute dichloromethane and 1.5 ml ether. This solution was added to 2.5 ml of a dried ether solution of diazomethane, freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide ** by conventional methods [5]. Analytically pure 26,27-bisnor-25-diazo-Δ^5- cholesten-3β-ol-24-one acetate (**4**) was obtained by preparative thin-layer chromatography (TLC) on a Silica Gel G *** layer, 1 mm thick, with dichloromethane-acetone (24 : 1). Yield : 38.5 mg (75.9 %); m.p. 155-158° d; lit. m.p. 158-160° d [6], 153° d. [7].

Nuclear magnetic resonance (NMR) spectra **** showed sharp signals of the C-18 and C-19 methyl groups (C-18, singlet at 0.68 ppm; C-19, singlet at 1.02 ppm). The protons of the C-21 methyl group appeared as a doublet at 0.90 ppm ($J \sim 5$ cps). The sharp singlet signal at 2.01 ppm must be attributed to the 3β-acetate group, whereas the doublet at 5.37 ppm ($J \sim 4$ cps) must be assigned to the vinylic proton at C-6, and the broad band at ~ 4.60 ppm to the α-proton at C-3. Finally, the proton at C-25 (diazomethyl group) evokes the sharp signal at 5.20 ppm.

*3β-Hydroxy-Δ^5-homocholenic acid phenyl ester acetate (**5**) from 26,27-bisnor-25-diazo-Δ^5-cholesten-3β-ol-24-one acetate (**4**).*

An improved procedure [8] was used for the Arndt-Eistert rearrangement of the diazoketone **4**. Twenty mg (0.0455 mmole) of the diazoketone **4** was decomposed by treatment with 100 mg (1.060 mmole) phenol and 0.1 ml *N,N*-dimethylaniline at 160-170 °C for 5 min until the evolution of nitrogen had stopped. The crude phenyl ester **5** was worked up by the procedure described

* All melting points were determined on a Kofler block and are corrected.

** Diazald, purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

*** Silica Gel G plates were purchased from Analtech, Inc., Wilmington, Del.

**** NMR spectra (60 MHz) were taken on a Varian Analytical Spectrometer, Model A-60A, in $CDCl_3$. Chemical shifts are indicated in ppm with $SiMe_4$ as internal standard.

above. Isolation by preparative TLC as before with CH_2Cl_2 gave 5.0 mg (21.7 %) analytically pure crystalline 3β-hydroxy-Δ^5-homocholenic acid phenyl ester acetate (**5**), m.p. 125-129 °C. Analysis for $C_{33}H_{46}O_4$ (506.7) : calcd., C 78.42 %, H 9.15 %; found, C 78.72 %, H. 9.26 %.

The NMR spectrum of the phenyl ester acetate (**5**) clearly showed that the signal at 5.20 ppm (C-25 proton of **4**) was absent, but proton signals of the aromatic system were recognizable as a multiplet between 7.1 ppm and 7.4 ppm ($J \sim 12$ cps). As expected, no change of chemical shift occurred in the other signals, described for the diazoketone **4**.

*25-Hydroxycholesterol-3-acetate (**7**) from 3β-hydroxy-Δ^5-homocholenic acid phenyl ester acetate (**5**).*

To a Grignard reagent, freshly prepared from 15.0 mg (0.106 mmole) CH_3I and 20 mg Mg turnings (cut in small pieces) in 1.0 ml dry ether, 10.0 mg (0.0198 mmole) 3β-hydroxy-Δ^5-homocholenic acid phenyl ester acetate (**5**), dissolved in 0.5 ml ether, was added. The mixture was refluxed for 2 hrs, then poured on ice, acidified with 2*N* H_2SO_4, and worked up as before. The crude product was isolated by preparative TLC, as before, yielding 3.3 mg (41.1 %) of pure 25-hydroxycholesterol (**6**); m.p. 177-178.5°; lit. m.p. 181.5-182.5 °C [8] and 177-179 °C [9].

Acetylation of the 25-hydroxycholesterol (**6**) by the procedure described above gave 25-hydroxycholesterol 3-acetate (**7**) in 90 % yield; m.p. 141-142 °C; lit. m.p. 142-142.8 °C [8], 138.5-140 °C [9]. The NMR spectra of **6** and **7** showed a sharp singlet at 1.20 ppm due to the C-26 and C-27 terminal methyl groups. As expected, the other significant bands were unchanged (CH_3COO-, 2.01 ppm; C-18, 0.68 ppm; C-19, 1.02 ppm; > CHOR, 4.60 ppm; > C = CH-, 5.37ppm).

*25-Bromocholesterol acetate (**8**) from 25-hydroxycholesterol 3-acetate (**7**).*

The bromination was carried out as described by Dauben *et al.* [9, 10]. To a cooled solution of 26.5 mg (0.059 mmole) 25-hydroxycholesterol 3-acetate (**7**) in 1.0 ml benzene 0.15 ml dry PBr_3 was added. The mixture was refluxed for 5 hrs and then worked up as before. The crude 25-bromocholesterol acetate (**8**) was recrystallized from ether-acetone, dried, and stored under nitrogen. Yield : 27.0 mg (89.5 %); m.p. 112-114 °C, lit. m.p. 113.5-115 °C [10, 11].

The NMR spectrum of **8** showed, among other signals, the sharp singlet due to the terminal C-26 and C-27 methyl groups, shifted to 1.72 ppm due to the introduction of the bromine atom at C-25.

*Cholesterol-25-^{3}H (**9**) from 25-bromocholesterol acetate (**8**).*

To a cooled solution of 35.0 mg (0.069 mmole) 25-bromocholesterol acetate (**8**) in 1.0 ml anhydrous ether (dried over sodium) 9.5 mg (0.206 mmole)

$LiAl^3H_3$* was added. The mixture was stirred well and refluxed for 1 hr. Following the usual work-up, the crude cholesterol-25-^{3}H (**9**) was purified by preparative TLC with dichloromethane-acetone (9 : 1) and a second TLC with cyclohexane-ethyl acetate (4 : 1) **. No radioactive impurities were observed. Crystallization from aqueous methanol then gave 5.0 mg (18.9 %) of chromatographically pure cholesterol-25-^{3}H (**9**); m.p. 147-5-148 °C, lit. m.p. 148.5 °C [12]. The NMR spectrum of a nonradioactive sample, prepared in the same manner, was identical to that of authentic cholesterol.

The radioactivity *** of the synthetic cholesterol-25-^{3}H (**9**) was $1.41.10^7$ dpm/mg or $5.42.10^9$ dpm/mmole. A portion of the synthetic cholesterol-25-^{3}H (**9**) was diluted with carrier cholesterol and recrystallized twice from aqueous methanol without change in specific activity (Table I).

*Δ^5-Androsten-3β-ol-17-one semicarbazone acetate (**10**) from cholesterol-25-^{3}H (**9**)* [13].

Thirty mg (0.078 mmole, 5,400 cpm/mg) cholesterol-25-^{3}H (**9**) was acetylated with 1.5 ml acetic anhydride and 1.5 ml pyridine and worked up as described earlier. Isolation by preparative TLC, as before, gave 30.1 mg (91 %) of pure cholesterol-25-^{3}H acetate; m.p. 113-114 °C, lit. m.p. 115 °C [14]. The pure cholesterol-25-^{3}H acetate was dissolved in 0.3 ml dichloromethane-ether (1 : 3), cooled to 10 °C, buffered with a solution of 5.0 mg sodium acetate in 0.5 ml aqueous acetic acid, and treated under stirring with an excess of a bromine-glacial acetic acid solution. The resulting 5,6-dibromocholestan-3β-ol-25-^{3}H acetate was separated by filtration and washed with acetic acid. The moist filter cake was taken up in 1.0 ml acetic acid and oxidized at 0-5 °C by dropwise addition over a period of 3 hrs of 5.0 ml of a solution, prepared by dissolving 3.96 g CrO_3 in 3.60 ml concentrated H_2SO_4, 5.00 ml H_2O, and 12.00 ml CH_3COOH according to the literature [15]. The excess of unreacted chromic acid was then reduced by the dropwise addition of 0.3 ml methanol, the mixture was repeatedly extracted with 10-ml portions of dichloromethane-ether (1 : 4), and the combined extracts were evaporated to a small volume.

Some zinc dust was added to the solution, the mixture was stirred for 1 hr, taken up in 2 ml H_2O, and then worked up as described earlier. When treated with semicarbazide acetate in methanol in the usual manner, the semicarbazone of Δ^5-androsten-3β-ol-17-one acetate (**10**) was obtained. Preparative TLC with benzene-ether (19 : 1), gave chromatographically pure

* $LiAl^3H_4$, having a specific activity of 100 mc per mmole, was purchased from New England Nuclear Corporation.

** Radiochromatograms were scanned on a Packard Radiochromatogram Scanner, Model 7201.

*** Aliquots of radioactive samples were counted in a liquid scintillation spectrometer, either the Nuclear Chicago Model 725 (efficiency 24.6 %) or the Packard Tri-Carb Model 3003 (efficiency 31.4 %).

Δ^5-androsten-3β-ol-17-one semicarbazone acetate (**10**). Yield : 1.75 mg (5.8 %); m.p. 269-273° d., lit. m.p. 274-276° d.[16]. No radioactivity was detected in this product.

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