SYNTHESIS OF Δ^7-COPROSTENOL AND ITS IDENTITY WITH THE STEROL FORMED BY THE REDUCTION OF 7-DEHYDROCHOLESTEROL BY INTESTINAL MICROORGANISMS

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ABSTRACT

A method for the preparation of Δ^7-coprostenol and the confirmation of its identity as the sterol formed by the reduction of 7-dehydrocholesterol by intestinal microorganisms has been presented. The $\Delta^{8(14)}$- and Δ^{14}-coprostenols were also prepared. Comparative gas-liquid chromatographic and thin-layer chromatographic data on the 5α and 5β Δ^7-, $\Delta^{8(14)}$- and Δ^{14}-cholestenols and their acetates are also given.

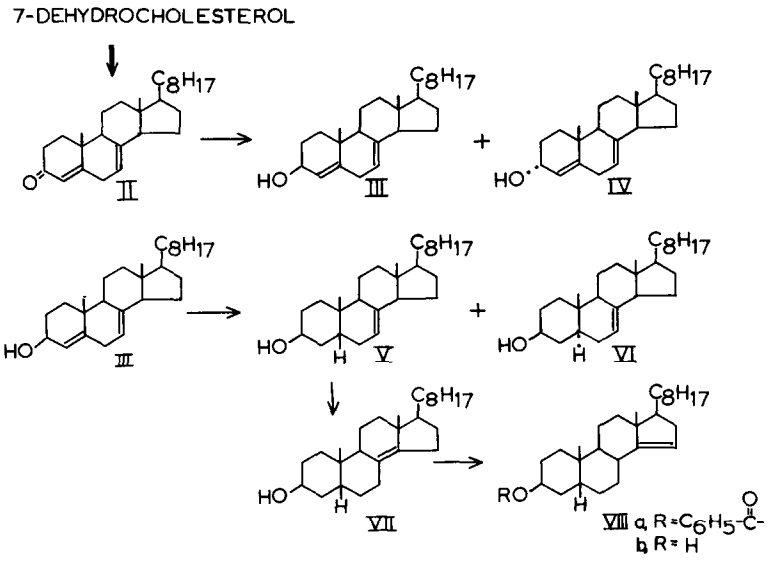
Δ^7-coprostenol (5β-cholest-7-en-3β-ol) has been identified as a minor constituent of the sterol of rat feces (1) and as the major sterol isolated from an incubation medium containing 7-dehydrocholesterol and inoculum derived from the large intestine of rats (2). The identification of Δ^7-coprostenol was based primarily on its catalytic reduction to coprostanol and molecular rotation studies and, to our knowledge, the compound has not been prepared synthetically.

Our immediate need for Δ^7-coprostenol and derivatives in our insect nutritional and hormonal studies prompted us to prepare the material. This paper describes a convenient procedure for the preparation of Δ^7-coprostenol. The $\Delta^{8(14)}$- and Δ^{14}-coprostenols were also prepared via Δ^7-coprostenol. In addition, comparative gas-liquid chromatographic (GLC) data on the 5α and 5β Δ^7-, $\Delta^{8(14)}$-, Δ^{14}-cholestenols and their acetates on three systems are presented.

An Oppenauer oxidation of 7-dehydrocholesterol (I) gave, after chromatography on silica gel, the cholesta-4,7-dien-3-one (II) in 56% yield. An ultraviolet absorption maximum at 230 mμ, ε 17,400 (in hexane), is indicative of the 3-keto-Δ^4 chromophore. Also, the infrared absorption bands at 1675 and 1625 cm^{-1} are in agreement with such a structure. The reduction of II with sodium borohydride or lithium aluminum hydride gave a 60:40 mixture of cholesta-4,7-dien-3β and 3α-ol, (III and IV), respectively, and they were inseparable by column chromatography on several different adsorbents (silica gel, Woelm, activity grade II neutral alumina and Florisil). Interestingly, a lithium aluminum hydride reduction of cholest-4-en-3-one under similar conditions gave cholest-4-en-3β-ol in 86% yield. The mixture of III and IV was separable by thin-layer chromatography (TLC) though it was not possible to separate the two compounds by preparative TLC because of the mixture's rapid crystallization on the plate; thus, the compounds were separated through their digitonides. The catalytic hydrogenation of cholesta-4,7-dien-3β-ol with platinum oxide in ethyl acetate gave Δ^7-coprostenol (V) and Δ^7-cholestenol (VI) in yields of 73% and 16%, respectively. Only slight hydrogenolysis occurred, as indicated by formation of hydrocarbon in 6% yield.

A direct comparison by infrared, GLC, and TLC analyses of the synthetic Δ^7-coprostenol and the sterol that we isolated from the incubation of 7-dehydrocholesterol with an inoculum from rat intestine according to the previously reported method (1) showed the two compounds to be identical.

The $\Delta^{8(14)}$-coprostenol (VII) was prepared in yield of 78% by shaking Δ^7-coprostenol with hydrogen and platinum oxide in acetic



acid, according to the method of Wieland et al. (3). The 3,5-dinitrobenzoate of VII exhibited physical properties similar to those of the 3,5-dinitrobenzoate of $\Delta^{8(14)}$-coprostenol prepared previously (4) by the catalytic hydrogenation of the acetate of 5β-cholesta-6,8-dien-3β-ol.

The isomerization of the benzoate of VII with hydrogen chloride in chloroform at -30° followed by saponification yielded the Δ^{14}-coprostenol (VIIIb).

We hoped that gas-liquid chromatography would be a valuable tool for the detection of the Δ^{7}-, $\Delta^{8(14)}$-, Δ^{14}-coprostenols or the corresponding cholestenols in biological systems. Thus, we compared the relative retention time of these unsaturated sterols on three commonly used GLC systems, SE-30, QF-1, and NGS. The results are given in table 1. In the coprostene series, neither the sterols nor their acetates were separated from each other by more than one minute. In the cholestene series, the Δ^{7}-sterol can be readily separated from the $\Delta^{8(14)}$- and Δ^{14}-

Table 1. GLC analysis of synthetically prepared coprostenols, cholestenols, their acetates, and Δ^7-coprostenol from microbiological reduction of 7-dehydrocholesterol.

Compounds	Relative retention times [a]		
	SE-30 [b]	QF-1 [c]	NGS [d]
Δ^7-Coprostenol	1.69	2.40	5.36
Δ^7-Coprostenol (from *in vitro*)	1.69	2.40	5.39
$\Delta^{8(14)}$-Coprostenol	1.71	2.35	5.21
Δ^{14}-Coprostenol	1.58	2.21	5.18
Δ^7-Cholestenol	2.07	3.22	7.60
$\Delta^{8(14)}$-Cholestenol	1.83	2.82	6.16
Δ^{14}-Cholestenol	1.83	2.87	6.73
Δ^7-Coprostenol acetate	2.29	3.98	4.68
Δ^7-Coprostenol acetate (from *in vitro*)	2.29	3.97	4.68
$\Delta^{8(14)}$-Coprostenol acetate	2.29	3.85	4.65
Δ^{14}-Coprostenol acetate	2.14	3.64	4.62
Δ^7-Cholestenol acetate	3.07	4.96	7.04
$\Delta^{8(14)}$-Cholestenol acetate	2.67	4.29	5.68
Δ^{14}-Cholestenol acetate	2.68	4.42	6.14

a/Relative to cholestane.

b/Column 6 ft x 4 mm ID, 0.75% SE-30 on 100-140 mesh, Gas-Chrom P, 16 psi, 232° C, cholestane time 4.95 min.

c/Column 6 ft x 4 mm ID, 3% QF-1 on 100-140 mesh, Gas-Chrom P, 38 psi, 216° C, cholestane time 5.62 min.

d/Column 6 ft x 4 mm ID, 0.75% neopentyl glycol succinate on 100-140 mesh, Gas-Chrom P, 34 psi, 214° C, cholestane time 2.5 min.

sterols; however, the latter two were not separable from each other to any appreciable extent.

The Δ^{14}-sterol of both series can be easily separated by TLC from the Δ^7-and $\Delta^{8(14)}$-sterols (table 2), but we have not succeeded in numerous attempts to develop a TLC or GLC system that will separate a mixture of all three sterols of the 5α- and 5β- series into their respective pure components.

Table 2. TLC analysis of coprostenols and cholestenols and their acetates

Compounds	R_f values [a]	Acetates R_f values [b]
Δ^7-Coprostenol	0.36	0.39
$\Delta^{8(14)}$-Coprostenol	0.36	0.45
Δ^{14}-Coprostenol	0.38	0.09
Δ^7-Cholestenol	0.23	0.49
$\Delta^{8(14)}$-Cholestenol	0.26	0.39
Δ^{14}-Cholestenol	0.25	0.09

a/Layer: Silica Gel H impregnated with Rhodamine 6G; development distance; 15 cm; solvent system: benzene - ethyl acetate (9:1).

b/Layer: Silica Gel H impregnated with 20% silver nitrate; development distance: 15 cm; solvent system: hexane - benzene (5:3).

A convenient method for the preparation of Δ^7-coprostenol and the confirmation of its identity as the sterol metabolite from the reduction of 7-dehydrocholesterol by intestinal microorganisms has been presented. The comparative GLC data, which should be of value to those interested in the sterols present in biological systems, indicate that while GLC and TLC will readily differentiate between the 5α- and 5β- cholestenol series, the complete separation of the Δ^7-, $\Delta^{8(14)}$-, and Δ^{14}-sterols of either series by GLC or TLC is difficult to attain.

Whether the $\Delta^{8(14)}$- and Δ^{14}-sterols of either the 5α- or 5β- series will be found in biological systems remains to be seen. However, the ready availability of these compounds through synthesis, together with a knowledge of their physical properties and their behavior on three GLC systems and on TLC, should rapidly enhance their detection and identification.

EXPERIMENTAL

All melting points were determined on the Kofler (5) block. Rotations were determined in approximately 1% solutions in chloroform at 23°. Infrared spectra were obtained in CS_2 with a Perkin-Elmer model 221 prism-grating double beam spectrophotometer. Gas-liquid chromatography analyses were made on Barber-Colman models 10 and 15. A radium sulfate ionization source was used in the detector cell, and argon was the carrier gas. The inert support was prepared and coated according to the method of Horning et al. (6), and the gas-liquid chromatography systems used were SE-30, QF-1, and NGS.

Cholesta-4,7-dien-3-one (II) - An Oppenauer oxidation of 6.0 g of 7-dehydrocholesterol (purchased from Nutritional Biochemical Corp.) with 17.8 ml of cyclohexanone, 122 ml of toluene, and 1.63 g of aluminum isopropoxide proceeded as described for ergosterol (7). The 6 g of crude product was chromatographed over 90 g of hexane-washed silica gel. The column was eluted with 500 ml of hexane, and thirteen 100 ml fractions of 5% ether-hexane were collected. Those 5% ether-hexane fractions with only the carbonyl absorption bands at 1675 and 1625 cm^{-1} were combined and recrystallized from acetonitrile to give 3.36 g of cholesta-4,7-dien-3-one (II), m.p. 71-73° resolidifies, m.p. 87-89°, αD +33, λ max 230 mμ in hexane, ϵ17,400 ν in CS_2 1675 cm^{-1} (carbonyl in conjugation with double bond) 1625 cm^{-1} (double bond in conjugation with carbonyl) (Lit. (8) m.p. 86-88°, λ max 238 mμ in ethanol 15,500).

Cholesta-4,7-dien-3β-ol (III) - To a solution of 30 ml of benzene and 60 ml of methanol at 5° was added 3.35 g cholesta-4,7-dien-3-one (II) and 0.6 g of sodium borohydride. The mixture was kept at 4° for 22 hr, 50 ml of water was added, and the methanol-benzene was removed in vacuo. The precipitate was collected, washed with water, and dried to give 3.4 g of material. A TLC showed two compounds that were inseparable by column chromatography. The compounds could be separated through their digitonides. To the 3.4 g of material dissolved in 500 ml of 80% ethanol at 80° was added 500 ml of hot 80% ethanol which contained 13.73 g of digitonin. The mixture was allowed to stand for 4 hr at room temperature, and the precipitate was collected and washed with 80% ethanol. The precipitated digitonide in 70 ml of pyridine was heated at reflux temperature for 45 min. Most of the pyridine was removed in vacuo, the material was triturated with ether, and the precipitate was collected and soxhlet extracted with ether. The ether solutions were concentrated to dryness, and the residues were examined by TLC. Only one compound could be detected. The residues were combined and recrystallized from ether-acetonitrile to give 1.28 g of cholesta-4,7-dien-3β-ol (III), m.p. 121-123°, αD +2.0°.

Anal. Calcd. for $C_{27}H_{44}O$: C, 84.31; H, 11.53; Found: C 84.53; H, 11.33.

The acetate (acetic anhydride-pyridine, 18 hr, 25°) was obtained as rectangular plates from ether-methanol, m.p. 107-110°, αD -31°.

Anal. Calcd. for $C_{29}H_{46}O_2$: C, 81.63; H, 10.87. Found: C, 81.65 H, 10.58.

The 3,5-dinitrobenzoate - (3,5-dinitrobenzoyl chloride-pyridine 18 hr. 25°) was recrystallized from ether-ethanol, m.p. 147-150° αD -30°.

Anal. Calcd. for $C_{34}H_{46}O_6N_2$: C, 70.56; H, 8.01. Found: C, 70.44; H, 7.67.

Cholesta-4,7-dien-3α-ol (IV) - The ethanolic filtrate, after separation of the insoluble digitonide in the preparation of III, was concentrated to dryness in vacuo. The digitonide residue processed in the same manner as the insoluble digitonide gave, after recrystallization from acetonitrile, 0.88 g of the cholesta-4,7-dien-3α-ol (IV), m.p. 91-94°, αD +49°.

Anal. Calcd. for $C_{27}H_{44}O$: C, 84.31; H, 11.53. Found: (9).

The acetate (acetic anhydride-pyridine, 18 hr, 25°) was recrystallized from ether-methanol, m.p. 97-99°, αD +133°.

Anal. Calcd. for $C_{29}H_{46}O_2$: C, 81.63; H, 10.87. Found: C, 81.84; H, 10.81.

The 3,5-dinitrobenzoate (3,5-dinitrobenzoyl chloride-pyridine, 18 hr 25°) was recrystallized from ether-ethanol, m.p. 144-147°, αD +151°.

Anal. Calcd. for $C_{34}H_{46}O_6N_2$: C, 70.56; H, 8.01. Found: C, 70.79; H, 7.93.

5β-Cholest-7-en-3β-ol (V) - A solution of 1 g of cholesta-4,7-dien-3β-ol, 75 ml of ethyl acetate and 0.1 g of platinum oxide was shaken with hydrogen at room temperature and atmospheric pressure for 2 hr, at which time slightly more than one molecular equivalent of hydrogen had been consumed. The catalyst was removed by filtration, and the solution was concentrated to dryness in vacuo. A GLC analysis showed the material to contain hydrocarbons and one major and two other minor compounds. The material was chromatographed on 30 g of hexane-washed alumina (activity grade II). The first fraction eluted with 100 ml of hexane gave hydrocarbons in 6% yield; then six 100 ml fractions of hexane-benzene (1:1) were collected. The fractions monitored by GLC on SE-30, which showed only one peak and exhibited an RRT of 1.69, were combined and recrystallized from ether-acetonitrile to give 730 mg of 5β-cholest-7-en-3β-ol (V), m.p. 104-106°, αD +49°, (Lit. (2), m.p. 105-106°, αD +54° ± 4°).

Further elution of the column with ether yielded 160 mg of Δ^7-cholestenol (VI). The product was identical (m.p., rotation, infrared spectrum) with an authentic sample of Δ^7-cholestenol.

The acetate of V (acetic anhydride-pyridine 18 hr, 25°) was recrystallized from ether-methanol, m.p. 88-90°, αD +50°, (Lit. (2), m.p. 90-91°, αD +48° ± 3°).

The benzoate of V (benzoyl chloride-pyridine, 18 hr, 25°) was

obtained as spears from acetone-methanol, m.p. 128-129°, αD +59°, (Lit. (2), m.p. 128-129°, αD +56° ± 3°).

Δ7-Coprostenol from Incubation of 7-dehydrocholesterol in vitro - An incubation medium of 250 mg of 7-dehydrocholesterol, 5 g of freshly prepared sterol-free dried beef brain, 100 ml of specially prepared medium, and 10 ml of an inoculum (content of large intestine of rat) was prepared, incubated for 7 days, and worked up as previously described by Coleman and Baumann (1). The ether extractable material was chromatographed over 15 g of hexane-washed alumina (activity grade II). The following fractions were collected: 100 ml of hexane and ten 50 ml fractions of hexane-benzene (1:1). The fractions were monitored by GLC on an SE-30 column programmed from 150-235°; the fractions showing only one compound, were combined and concentrated to dryness in vacuo (10). Recrystallization from ether-acetonitrile gave 100 mg of spears, m.p. 103-105°, αD +53°.

The acetate (acetic anhydride-pyridine, 18 hr, 25°) gave plates m.p. 88-90°, αD +51°.

The Δ7-coprostenol prepared synthetically and the Δ7-coprostenol obtained from in vitro and their respective acetates gave identical infrared spectra and identical RRT on three different GLC systems (table 1).

5β-Cholest-8(14)-en-3β-ol (VII) - a solution of 0.426 g of 5β-cholest-7-en-3β-ol (V), 0.043 g of platinum oxide, and 10 ml of acetic acid was shaken with hydrogen at room temperature and atmospheric pressure for 2 hr. The catalyst was removed by filtration and the solvent removed in vacuo. An infrared analysis showed the absorption bands at 3015 and 836 cm^{-1}, indicative of the Δ7(8)-double bond, were no longer present. The compound recrystallized from acetone-acetonitrile yielded 334 mg, m.p. 83-85°, αD +33°.

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found: (9).

The benzoate of VII, a mixture of 0.36 g of VII, 8 ml of pyridine, and 0.5 ml of benzoyl chloride at 25° for 18 hr, yielded, after recrystallization from acetone-methanol, 278 mg of the benzoate, m.p. 96-98°, αD +32°.

Anal. Calcd. for $C_{34}H_{50}O_2$: C, 83.20; H, 10.13. Found: C, 83.43, H, 10.05.

The 3,5-dinitrobenzoate of VII (3,5-dinitrobenzoyl chloride-pyridine, 18 hr, 25°) was recrystallized from acetone-methanol, m.p. 186-190°, αD +32°, (Lit. (3), m.p. 181°, αD +27°).

5β-Cholest-14-en-3β-ol benzoate (VIIIa) - A gentle stream of dry hydrogen chloride gas was passed through a solution of 0.267 g of Δ8(14)-coprostene-3β-ol benzoate and 2 ml of chloroform for 2 hr at -30°. A slight vacuum was then applied to the side arm of the test tube to remove the excess hydrogen chloride gas. The solution was poured gently into 10 ml of 5% sodium bicarbonate solution and extracted

with ether. The ether phase was washed with water, dried over sodium sulfate, and concentrated to dryness in vacuo. The compound recrystallized from acetone-methanol yielded 0.125 g of 5β-cholest-14-en-3β-ol benzoate, m.p. 88-90°, αD +47°.

Anal. Calcd. for $C_{34}H_{50}O_2$: C, 83.20; H, 10.13. Found: C, 82.96; H, 9.86.

5β-Cholest-14-en-3β-ol (VIIIb) - A mixture of 0.12 g of the benzoate VIIIa, 10 ml of benzene, 25 ml of methanol, and 3.5 g of potassium hydroxide was refluxed for 2 hr. The solvent was removed in vacuo, water was added, and the precipitate was collected. Recrystallization from acetone-acetonitrile gave 50 mg of VIIIb as needles, m.p. 113-115°, αD +27°, νmax. in CS_2 3050 cm^{-1} (Δ^{14}-double bond).

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found: (9).

The 3,5-dinitrobenzoate of VIIIb (3,5-dinitrobenzoyl chloride-pyridine 18 hr, 25°) was recrystallized from ether-methanol, m.p. 188-191°, αD +28°.

Anal. Calcd. for $C_{34}H_{48}O_6N_2$: C, 70.32; H, 8.33. Found: C, 70.20; H, 8.27.

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9. We were unable to obtain carbon and hydrogen analyses within the required ± 0.3% for compounds IV, VII and VIIIb. However, their derivatives gave good elemental analyses, and the original compounds, when analyzed by GLC and TLC, did not show any impurities.

10. The earlier hexane-benzene (1:1) fractions contained a crystalline long-chain alcohol.