Isolation and chemical characterization of $\Delta^{5,7,24}$-cholestatrien-3β-ol from pig tissues

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ABSTRACT Although indirect evidence has implicated $\Delta^{5,7,24}$-cholestatrien-3β-ol as a possible intermediate in cholesterol biosynthesis, this sterol has not previously been isolated from tissues. Administration of two inhibitors of cholesterol biosynthesis to pigs led to the accumulation of $\Delta^{5,7,24}$-cholestatrien-3β-ol in the tissues, and this sterol was isolated from the lung. Proof of its chemical identity was based upon UV, IR, NMR, circular dichroism, and mass spectra, as well as comparison with synthetic $\Delta^{5,7,24}$-cholestatrien-3β-ol.

A fragment at m/e 143 is particularly prominent in the mass spectrum of $\Delta^{5,7}$-sterols, and this fact may prove useful for the detection of this functional group. It is proposed that $\Delta^{5,7,24}$-cholestatrien-3β-ol may be an intermediate in sterol biosynthesis in both animals and plants.

SUPPLEMENTARY KEY WORDS cholesterol · biosynthesis · $\Delta^{5,7}$-sterols · mass spectra · circular dichroism

AVAILABLE EVIDENCE supports a role in cholesterol biosynthesis for both $\Delta^{5,7}$- and Δ^{24}-sterols[1] (1–6), and $\Delta^{5,7,24}$-cholestatrien-3β-ol (I, see Fig. 1) has therefore been proposed as a possible intermediate in cholesterol biosynthesis (7). Our demonstration (8) of the conversion of chemically synthesized $\Delta^{5,7,24}$-cholestatrien-3β-ol-3α-^{3}H to cholesterol in the rat, both in vivo and in vitro, directly supported this hypothesis.

Although the possible existence of a $\Delta^{5,7,24}$-sterol in biological material has been suggested by indirect evidence from several laboratories (9–13), the isolation and chemical characterization of this sterol from biological sources has not previously been reported. We describe here the isolation of this sterol from pigs treated with two inhibitors of cholesterol biosynthesis, AY-9944 [*trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] and 20,25-diazacholesterol (SC-12937). We thank Dr. D. Dvornik of Ayerst Laboratories for the AY-9944 and Dr. R. E. Ranney of G. D. Searle and Co. for the SC-12937.

MATERIALS AND METHODS

Isolation of $\Delta^{5,7,24}$-Cholestatrien-3β-ol (I)

For 3 weeks two weanling pigs received AY-9944, 20 mg/kg, and 20,25-diazacholesterol (SC-12937), 1 mg/kg, mixed with their regular diet. Sterols were isolated from both liver and lung tissue. A typical isolation procedure was as follows. 100 g of lung tissue was saponified in 500 ml of 15% ethanolic KOH at reflux in an atmosphere of nitrogen for 3 hr. After the reaction mixture had cooled to room temperature, 500 ml of water was added and the mixture was extracted with three 1 liter portions of petroleum ether. The petroleum ether was removed by a stream of nitrogen, and the residue was applied in benzene to a silicic acid column (14), 2.5 × 100 cm. 20-ml fractions were collected. The isolated sterols (peak at fraction 94, see Results) were further purified by additional silicic acid chromatography and finally by crystallization from either anhydrous methanol or acetone–water. The yield of $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) from 100 g of lung tissue was approximately 100 mg (0.1%).

Spectra

Infrared spectra were obtained from KBr pellets in a Perkin-Elmer 337 grating spectrometer.

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[1] Names of sterols used in this paper are: Δ^5-cholesten-3β-ol (cholesterol); $\Delta^{5,24}$-cholestadien-3β-ol (desmosterol); $\Delta^{5,7}$-cholestadien-3β-ol (7-dehydrocholesterol); $\Delta^{5,7,24}$-cholestatrien-3β-ol; $\Delta^{8,24}$-cholestadien-3β-ol (zymosterol); $\Delta^{7,24}$-cholestadien-3β-ol; epiperoxide of $\Delta^{5,7}$-cholestadien-3β-ol (5α,8α-epiperoxy-Δ^6-cholesten-3β-ol).

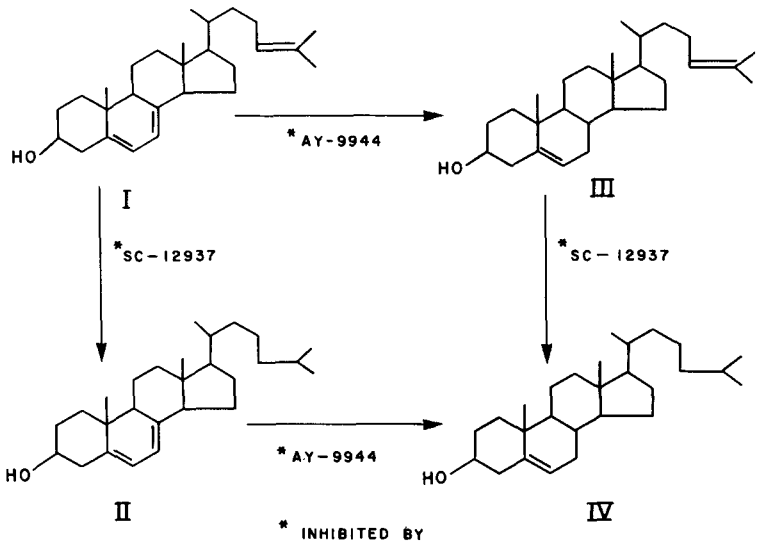

FIG. 1. Biosynthetic pathways involved in the conversion of $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) to cholesterol (IV).

Nuclear magnetic resonance (NMR) spectra were measured in a Varian A-60-A spectrometer with a 10% solution of sterol in $CDCl_3$.

Ultraviolet (UV) spectra were obtained with a Bausch & Lomb Spectronic 505 or Zeiss PMQ-II spectrometer with cyclohexane as solvent.

Circular dichroism (CD) spectra were obtained by using a Cary 60 spectropolarimeter with CD attachment and dioxane as solvent.

All mass spectral studies were performed with a CEC 21-110B mass spectrometer equipped with a combination detector system and an electronic peak-matching accessory for precise mass measurements. The samples were introduced into the ion source by the direct introduction probe technique. The ion source was held at 200°C, and the mass spectra were recorded on plates at probe temperatures of 150–160°C. Mass and isotopic abundance were measured on a sample introduced via the probe at 155°C. The photographic plates were scanned by means of a Jarrell-Ash model 24-300 recording microphotometer.

RESULTS

The first silicic acid chromatogram of the unsaponifiable extract is shown in Fig. 2. The largest peak (at tube 94) corresponds to $\Delta^{5,7,24}$-cholestatrien-3β-ol (I); 7-dehydrocholesterol (II) was incompletely separated from I and gave a peak at tube 86; cholesterol (IV) was present (peak at tube 80); and another sterol was found with a peak at tube 66. An IR spectrum of this peak was identical with that obtained for a sterol with the probable structure of 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol as described by Sanghvi (15). The peak corresponding to $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) was chromatographed twice more; Fig. 3 shows the silicic acid chromatogram obtained. An excellent correspondence between the Liebermann–Burchard color reaction (16) and the UV absorbance at 282 mμ is seen. This is important because it shows the absence of $\Delta^{7,24}$- and $\Delta^{8,24}$-sterols, which would have given a significant Liebermann–Burchard color reaction but would not have specific absorbance at 282 mμ. If these sterols had been present, they would have been detected on the more polar (right) side of the peak in Fig. 3.

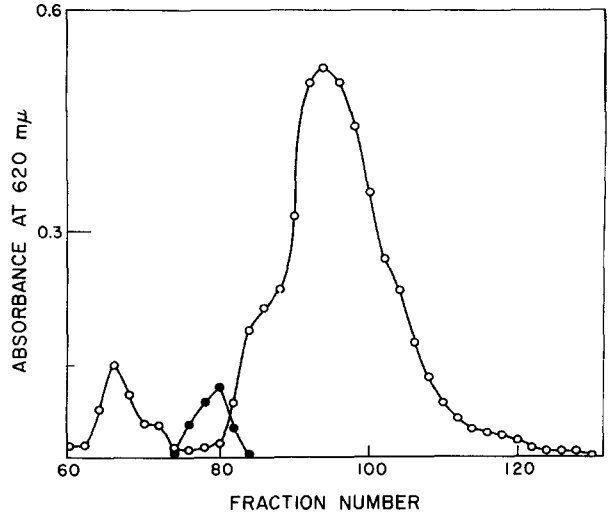

FIG. 2. First silicic acid chromatogram of the nonsaponifiable extract from the lungs of pigs treated with AY-9944 and 20,25-diazacholesterol (SC 12937). ○——○, Liebermann–Burchard color 1.5 min after addition of reagent; ●——●, Liebermann–Burchard color 30 min after addition of reagent.

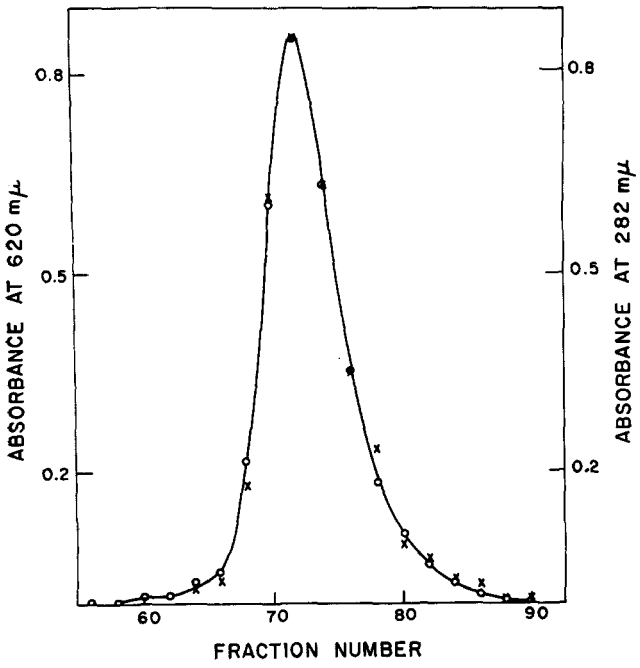

FIG. 3. Silicic acid chromatogram of $\Delta^{5,7,24}$-cholestatrien-3β-ol, isolated from the lungs of pigs treated with AY-9944 and 20,25-diazacholesterol (SC-12937), after third passage through silicic acid column. x——x, UV absorbance at 282 mμ; O——O, Liebermann–Burchard color 1.5 min after addition of the reagent.

The material whose elution pattern is shown in Fig. 3 was crystallized from anhydrous methanol; mp 107–108°C. The UV spectrum was typical of a steroid containing the $\Delta^{5,7}$-diene system (17); λ_{max} 294 mμ (ϵ = 6570), λ_{max} 282 mμ (ϵ = 11,200), and λ_{max} 271 mμ (ϵ = 10,300).

The NMR spectrum is shown in Fig. 4. Several pertinent observations can be made. (*a*) The presence of the Δ^{24}-bond is shown by peaks at δ 1.62 and 1.70 associated with the two isopropylidene methyls, C-26 and C-27 (18). (*b*) The C-6 and C-7 protons are seen as a quartet centered at δ 5.48. A similar quartet was seen in this region for 7-dehydrocholesterol. In addition, the C-24 proton is observed as a triplet centered at 5.12. (*c*) Integration of the region associated with olefinic protons revealed three protons attached to doubly bonded carbon in agreement with structure I. (*d*) A broad peak at δ 3.57 represents the 3α-proton. The location and configuration of this peak are consistent with an equatorial hydroxyl group at C-3 (19). (*e*) Calculations similar to those of Zürcher (20), which predict the expected resonant frequencies of the C-18 and C-19 angular methyl groups, were made: C-18 methyl, calculated δ 0.625, observed 0.637; C-19 methyl, calculated δ 0.950, observed 0.955. Also, the C-21 methyl protons were observed at δ 1.02 although separation from the C-19 methyl protons was incomplete.

Fig. 5 shows the IR spectra of I isolated from lung tissue and I prepared by chemical synthesis. These spectra are identical except for an additional small band at 885 cm^{-1} in the chemically synthesized material. This is caused by the presence of a trace amount of a [25]-impurity which, because it possesses a terminal methylene group, absorbs in this region (21). A decreased relative intensity of the band at 1365 cm^{-1} (when compared to the same band in the spectrum of 7-dehydrocholesterol) was noted and is consistent with the presence of the Δ^{24}-bond (18). Also, the location of the C–O stretching bands at 1040 cm^{-1} and 1064 cm^{-1} is similar to that seen with 7-dehydrocholesterol. This is important because the C–O stretching bands of the C-3 hydroxyl group are sensitive to the stereochemistry of the C-3 hydroxyl, the A/B ring juncture, and the presence and location of unsaturation in ring B (18). The Δ^{24}-bond, on the other hand, is remote from the C-3 hydroxyl and has little influence in this particular region (18).

The circular dichroism spectrum of I showed a series of strongly negative Cotton effects, seen at: $[\Theta]_{294}$ -1.55×10^6°; $[\Theta]_{282}$ -2.93×10^6°; $[\Theta]_{271}$ -3.13×10^6°; and a shoulder at $[\Theta]_{262}$ -2.36×10^6°. These troughs occur at essentially the same wavelengths as the UV absorption maxima and thus are associated with the $\Delta^{5,7}$-diene system. The negative values for the molecular ellipticity $[\Theta]$ in this region are consistent with a left-handed helicity of the $\Delta^{5,7}$-diene system (22), in agreement with the structure of I. The specific rotation, $[\alpha]_D$, obtained in chloroform, was −114°, essentially identical with our measured value for 7-dehydrocholesterol.[2]

Table 1 shows that the empirical formula of $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) isolated from the pig can be unambiguously identified as $C_{27}H_{42}O$, in agreement with structure I, from the precise mass measurement of the parent ion at m/e 382.4463. This assignment is based upon the assumption that I contains only carbon, hydrogen, and oxygen; this assumption was verified by both the mass spectrum of I and elemental analysis.

In addition, the ratio of m/e 384 to m/e 382 was observed to be 6.0×10^{-2}. A calculation (24) of this ratio, assuming the 382 peak to be due only to $C_{27}H_{42}O$ and the 384 peak only to isotopic contributions from $C_{27}H_{42}O$, resulted in a value of 4.5×10^{-2}. The difference is attributed to the presence of a trace amount of $C_{27}H_{44}O$, probably 7-dehydrocholesterol.

The high-resolution mass spectrum of $\Delta^{5,7,24}$-cholestatrien-3β-ol isolated from the pig was essentially identical with the spectrum obtained from $\Delta^{5,7,24}$-cholestatrien-

[2] The Δ^{24}-bond is far removed from both the $\Delta^{5,7}$-diene system (the primary contributor to this measurement) and the nearest asymmetric center (C-20). That the Δ^{24}-bond has essentially no effect upon the specific rotation, $[\alpha]_D$, has been confirmed experimentally (23).

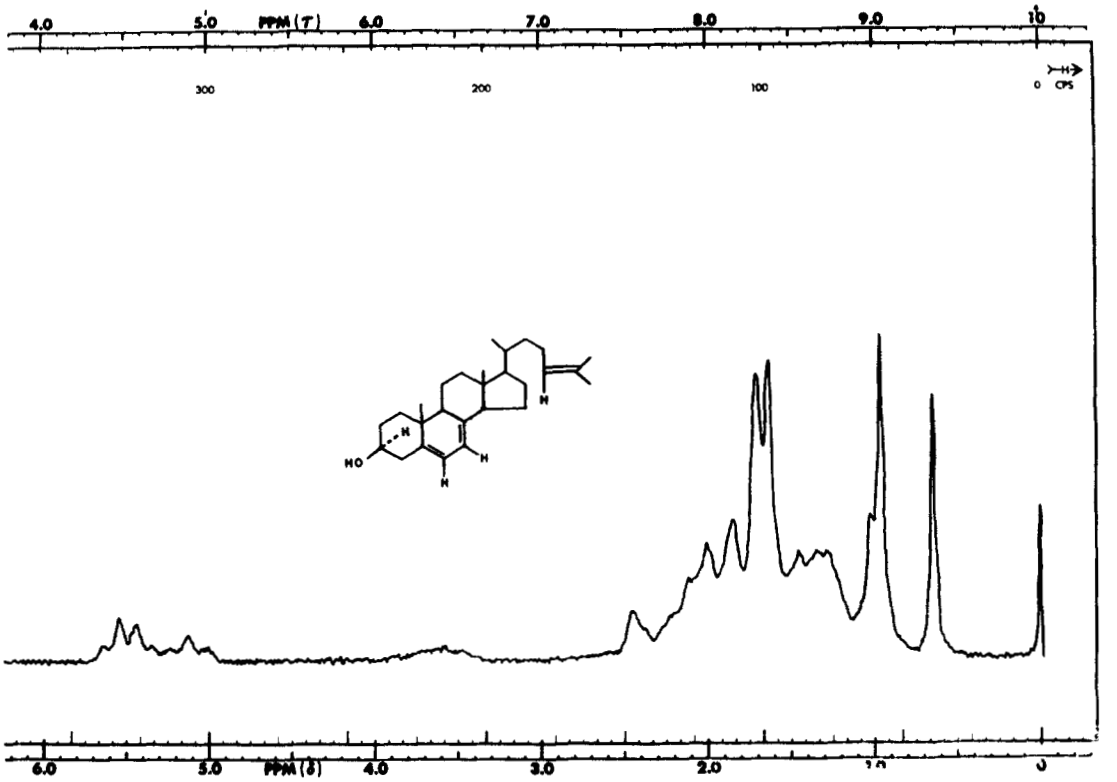

FIG. 4. NMR spectrum of $\Delta^{5,7,24}$-cholestatrien-3β-ol isolated from the lungs of pigs treated with AY-9944 and 20,25-diazacholesterol (SC-12937).

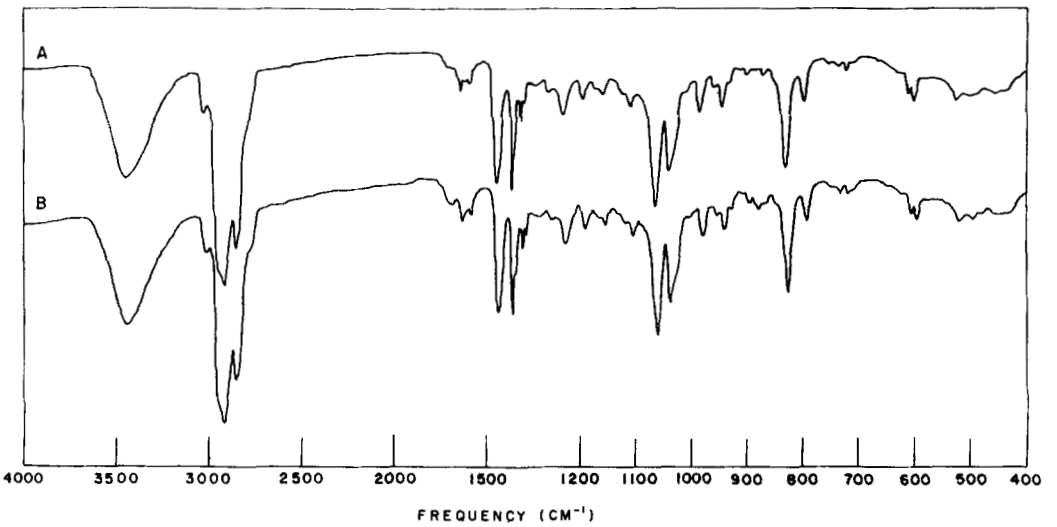

FIG. 5. IR spectrum of (A) $\Delta^{5,7,24}$-cholestatrien-3β-ol isolated from the lungs of pigs treated with AY-9944 and 20,25-diazacholesterol (SC-12937), and (B) $\Delta^{5,7,24}$-cholestatrien-3β-ol prepared by chemical synthesis.

3β-ol prepared by chemical synthesis. Table 2 and Fig. 6 illustrate the major fragmentations observed. The most intense peak observed above m/e 100 was at m/e 143. This is reasonable because of the probable aromatic character of this fragment. In addition, the mass spectrum of $\Delta^{5,7,24}$-cholestatrien-3β-ol shows a pronounced increase in the relative intensity of a peak at m/e 69 (compared to the same peak in the spectrum of 7-dehydrocholesterol). This difference is probably related to allylic cleavage of the side chain at C-22, C-23 in the case of $\Delta^{5,7,24}$-cholestatrien-3β-ol.

DISCUSSION

$\Delta^{5,7,24}$-cholestatrien-3β-ol (I) was first proposed as a possible intermediate in cholesterol biosynthesis by

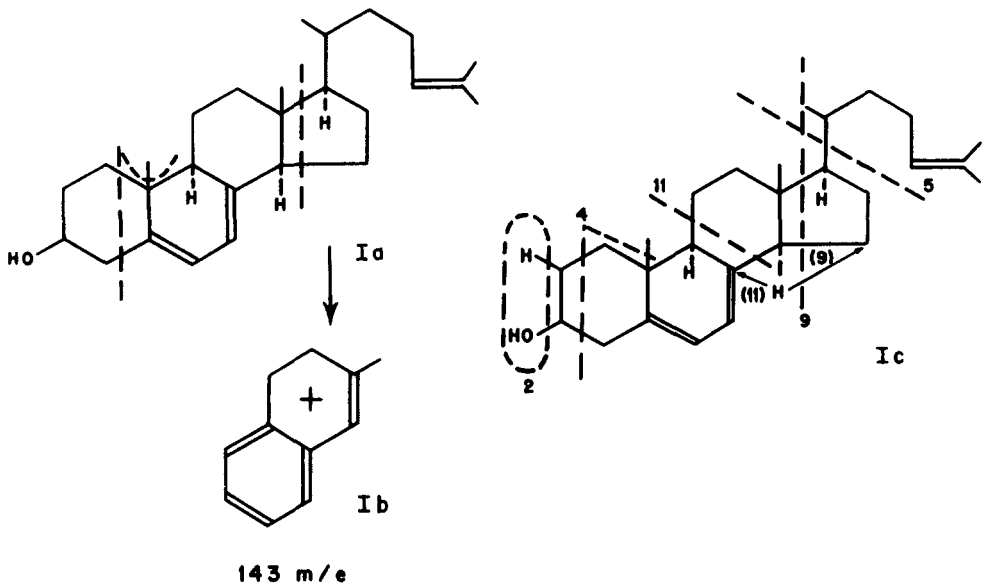

FIG. 6. Proposed fragmentations leading to mass spectrum of $\Delta^{5,7,24}$-cholestatrien-3β-ol. Numbers refer to Table 2, "Process."

Johnston and Bloch (7). This proposal resulted from their suggestion that $\Delta^{8,24}$-cholestadien-3β-ol (zymosterol) was an important intermediate in cholesterol biosynthesis.

A sterol with the possible structure of I was first detected by Frantz, Sanghvi, and Clayton (9) in the intestinal wall of triparanol-treated guinea pigs. Suggestion of the $\Delta^{5,7,24}$-structure was based upon chromatographic mobilities, the pharmacologic activity of triparanol as a Δ^{24}-reductase inhibitor, and biogenetic considerations. The presence of the $\Delta^{5,7}$-conjugated diene system was shown by the UV spectrum. Similar conclusions were reached by Galli and Maroni (12) and Fumagalli, Niemiro, and Paoletti (10). Dvornik, Kraml, and Bagli (13) reported detection of a sterol thought to be a $\Delta^{5,7,24}$-sterol by preparation of an epiperoxide derivative. This derivative was then converted by catalytic reduction to a presumed cholestane-3β,5α,8α-triol derivative. One difficulty in these experiments was that the preparations were contaminated with 7-dehydrocholesterol. This was due to the fact that (*a*) chromatographic techniques used to purify I or its epiperoxide derivative were unsuccessful, and (*b*) blockage of the Δ^{24}-reductase system by triparanol was incomplete. Thus 7-dehydrocholesterol would also form an epiperoxide derivative, and would also be converted by catalytic reduction to the presumed cholestane-3β,5α,8α-triol. Therefore, precise delineation of the side-chain structure by arguments similar to those presented here, such as the number of olefinic protons by NMR spectroscopy, was not possible. Dempsey (11), using a microsomal preparation from rat liver, has also reported detection of a radioactive compound thought to be a $\Delta^{5,7,24}$-sterol, formed from a biosynthetic substrate that was probably a mixture of $\Delta^{8,24}$- and $\Delta^{7,24}$-cholestadien-3β-ols. Assignment of the $\Delta^{5,7,24}$- structure was based upon formation of an epiperoxide, addition of the epiperoxide of 7-dehydrocholesterol, and catalytic reduction of this mixture to a presumed cholestane-3β,5α,-8α-triol derivative. Assignment of the Δ^{24}-bond was

TABLE 1 MASS MEASUREMENTS PERFORMED ON IONS IN SPECTRUM OF $\Delta^{5,7,24}$-CHOLESTATRIEN-3β-OL

m/e, Isolated Sterol	m/e, Standard	Mass, Isolated Sterol	Empirical Formula	Δ amu*	Mechanism
382	381(C_6F_{15})	382.4463†	$C_{27}H_{42}O$	+0.0012	Parent ion
			$C_{26}H_{38}O_2$	+0.0374	
			$C_{28}H_{46}$	−0.0350	
			$C_{29}H_{34}$	+0.0587	
229	231(C_5F_9)	229.2334	$C_{16}H_{21}O$	+0.0025	9‡
			$C_{17}H_{25}$	−0.0351	
			$C_{15}H_{17}O_2$	+0.0377	
211	205(C_6F_7)	211.2143	$C_{16}H_{19}$	−0.0004	12‡
			$C_{15}H_{15}O$	+0.0349	
			$C_{15}H_{31}$	−0.0954	
143	131(C_3F_5)	143.1320	$C_{11}H_{11}$	+0.0007	13‡
			$C_{10}H_7O$	−0.0368	
69	69(CF_3)	69.0922	C_5H_9	−0.0003	Allylic cleavage of side chain
			C_4H_5O	−0.0365	

* Difference in atomic mass units (observed mass minus calculated mass).

† Identical precise mass measurements were obtained for the parent ion of $\Delta^{5,7,24}$-cholestatrien-3β-ol isolated from pig lung and $\Delta^{5,7,24}$-cholestatrien-3β-ol prepared by chemical synthesis.

‡ See Table 2 for process.

TABLE 2 PREDOMINANT FRAGMENTATION PROCESSES OF $\Delta^{5,7,24}$-CHOLESTATRIEN-3β-OL*

Process	m/e of Fragment Ion+	Remainder		Abundance†
1	367	15	Loss of CH_3	xx
2	364	18	Loss of H_2O	xx
3	349	33	Loss of CH_3 + H_2O	xxxx
4	323	59	Loss of CH_3 + C_2H_4O	xxx
5	271	111	Loss of side chain (C_8H_{15})	xx
6	269	113	Loss of side chain + H_2	xx
7	253	129	Loss of side chain + H_2O	xxx
8	251	131	Loss of side chain + H_2O + H_2	xx
9	229	153	D ring cleavage with H transfer from ion	xx
10	227	155	Same as above + loss of H_2	xx
11	219	163	C ring cleavage with H transfer from ion	x
12	211	171	Loss of side chain + CH_3 + C_2H_5O‡	xxx
13	143	239	Loss of CH_3 + A and D ring cleavage‡	xxxx

* Molecular ion = 382 mass units, empirical formula = $C_{27}H_{42}O$. Bombardment was performed with 70 volt electrons, and the sample was introduced via the heated probe at 150–160°C with a source temperature of 200°C.

† Abundance of base peak (above m/e 100) = 100, xxxxx; abundance of parent peak, xxxx.

‡ Ion does not contain O.

based only upon chromatographic mobilities and biogenetic considerations. Since the presumed cholestane-3β,5α,8α-triol derivative was prepared by catalytic hydrogenation, the location and extent of unsaturation in the side-chain could not be determined.[3]

A further difficulty with these experiments (11, 13) is whether the derivative really was cholestane-3β,5α,8α-triol. Dvornik et al. (13) report the melting point for this compound as 185–187°C, whereas Dempsey (11) gives 207–208°C. This is a striking difference, yet both of these investigators relied upon the presumed structure of this derivative as evidence for the presence of a $\Delta^{5,7,24}$-sterol.

In the experiments reported here, we were able to isolate $\Delta^{5,7,24}$-cholestatrien-3β-ol directly in high purity. It was therefore possible to characterize the side-chain structure by NMR spectroscopy. Because the compound was pure, we could establish that three protons were attached to doubly bonded carbon and observe two peaks at δ 1.62 and 1.70 associated with the C-26 and C-27 isopropylidene methyl groups.

These facts, coupled with evidence presented elsewhere (18), allow the unambiguous assignment of the Δ^{24}-bond.

In addition, we were able to determine unambiguously for the first time the empirical formula of I as $C_{27}H_{42}O$ by high-resolution mass spectrometry. This removes any doubt concerning alternative structures which differ by addition or subtraction of CH_4 and O (see Table 1). Consideration of the mass spectrum may perhaps be a useful way of identifying $\Delta^{5,7}$-sterols. The fact that fragment Ib (Fig. 6), m/e 143, was the most intense peak above m/e 100 is reasonable because of the probable aromatic character of this ion. A similar finding was also made for 7-dehydrocholesterol. The mechanism shown in Fig. 6 (Ia → Ib) is only one of several possibilities. Which of the two angular methyls (C-18 or C-19) is preferentially cleaved cannot be decided until experiments are performed with the appropriate compounds containing deuterium at either C-18 or C-19. In addition, the possibility exists that Ib may possess a seven-membered aromatic ring. A similar proposal has recently been made by Galli and Maroni (12), but they were not able to unambiguously assign empirical formulas to the ions in question.

Of primary importance is that in the present study we have demonstrated that $\Delta^{5,7,24}$-cholestatrien-3β-ol isolated from pig lung and liver is identical with $\Delta^{5,7,24}$-cholestatrien-3β-ol prepared by chemical synthesis. This observation, combined with our earlier demonstration (8) of the biological conversion, both in vivo and in vitro, of chemically synthesized $\Delta^{5,7,24}$-cholestatrien-3β-ol-3α-^{3}H to cholesterol, suggests a possible intermediary role for this sterol in cholesterol biosynthesis.[4] $\Delta^{5,7,24}$-cholestatrien-3β-ol may also be an intermediate in ergosterol formation in yeast and sitosterol formation in plants. This proposal seems plausible because of recent work (25, 26) which shows that several Δ^{24}-sterols can be converted via enzymatic alkylation to ergosterol or sitosterol. Thus $\Delta^{5,7,24}$-cholestatrien-3β-ol could be an intermediate in sterol biosynthesis in both plants and animals.

[3] Another difficulty with previous studies (9, 11, 13) is that $\Delta^{5,7,24}$-sterols might have been formed as artifacts by dehydration of sterols, such as 3β,5α-dihydroxy-$\Delta^{7,24}$-cholestadiene or 3β-6-dihydroxy-$\Delta^{7,24}$-cholestadiene, that might have been initially present in the tissues or homogenates; these sterols might have undergone dehydration during saponification and chromatographic procedures to yield $\Delta^{5,7,24}$-sterols. We were able to exclude such artifacts as significant contributors: a chloroform–methanol 2:1 extract obtained at room temperature from the liver of a pig treated with AY-9944 and 20,25-diazacholesterol showed the UV spectrum characteristic of sterols containing the $\Delta^{5,7}$-system. Furthermore, sterols containing the $\Delta^{5,7}$-system accounted for 0.2% of the wet weight of the liver.

[4] Two pathways have been proposed (6) for the conversion of C_{27} sterols to cholesterol: one pathway emphasizes Δ^{24}-sterols as intermediates, e.g., demosterol (III in Fig. 1), while the other pathway emphasizes the corresponding compounds with saturated side-chains, e.g., 7-dehydrocholesterol (II in Fig. 1). For reasons discussed previously (6) it is not possible at present to decide which pathway quantitatively predominates. What can be said is that substantial evidence (1–7) exists for the operation of both pathways.

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