Characterization of the structure of a 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol isolated from rat skin

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ABSTRACT A new sterol has been isolated from the skin of rats treated with triparanol. Its chromatographic behavior on silicic acid-Celite columns and in gas–liquid chromatographic systems indicated it to be a 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol. The specific rotation, the delayed color reaction with Liebermann-Burchard reagent, and the nuclearma gnetic resonance (NMR) data support the $\Delta^{8(9)}$-unsaturation. Previous workers have shown that triparanol treatment results in an accumulation of Δ^{24}-unsaturated sterols in animal tissues. Consonant with this observation, the infrared, NMR, and mass spectrometric data confirm the presence of a C-24(25) unsaturated side chain in this sterol.

SUPPLEMENTARY KEY WORDS triparanol · nuclear magnetic resonance · gas–liquid chromatography · 4-methyl-$\Delta^{7,24}$-sterol · infrared spectra

CLAYTON, NELSON, AND FRANTZ (1) first detected the presence of a new sterol in the skin of rats treated with triparanol {MER-29, 1-(4-[diethylaminoethoxy]phenyl)-1-(p-tolyl)-2-(p-chlorophenyl) ethanol}, to which they assigned the tentative structure, 4α-methyl-$\Delta^{8,24}$-cholestadien-3β-ol. In this paper the isolation of this sterol is described, and data are presented to confirm the presence of the $\Delta^{8,24}$-diene system in it. From an interpretation of optical rotatory dispersion and mass spectrometric data, it has been shown in an earlier report (2) that the C-4 methyl group in this sterol actually has a β- rather than an α- configuration, contrary to the previous assumption.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing about 250 g and fed a diet of Purina Laboratory Chow were used throughout.

Chromatography of free sterols was performed as described by Frantz (3).

Columns of diameters from 1 to 10 cm and of lengths from 100 to 200 cm were used. The volume of fractions collected depended on the column dimensions. Colorimetric assay for "slow-" and "fast-acting" sterols was performed as suggested by Moore and Baumann (4).

The IR spectra were obtained on a Perkin-Elmer 521 IR spectrophotometer. NMR spectra were obtained on a Varian 100 Mc spectrometer at Varian (Palo Alto, Calif.). The resonance positions were mesaured relative to internal tetramethylsilane. The elemental analyses were performed by Aminco Laboratories, (Silver Springs, Md.). GLC of sterol methyl ethers was carried out by the method of Clayton (5). The operating conditions for GLC were the same as described by Frantz, Scallen, Nelson and Schroepfer (6).

EXPERIMENTAL PROCEDURE

48 female rats were maintained on a dosage of triparanol which was 10 mg/day per animal. The drug was dissolved in ether and was sprayed on rat chow using an atomizer; the ether was allowed to evaporate. The rats were killed after 3 months of drug treatment, and their skins were removed. The skins (960 g) were extracted with boiling acetone. The acetone extract (2550 ml) was concentrated to approximately 200 ml and was saponified for 6 hr with 2000 ml of 15% ethanolic KOH. The unsaponifiable material was extracted and isolated as previously described (1). The free sterols were chromato-

Abbreviations: NMR, nuclear magnetic resonance; IR, infrared; GLC, gas–liquid chromatography; ORD, optical rotatory dispersion.

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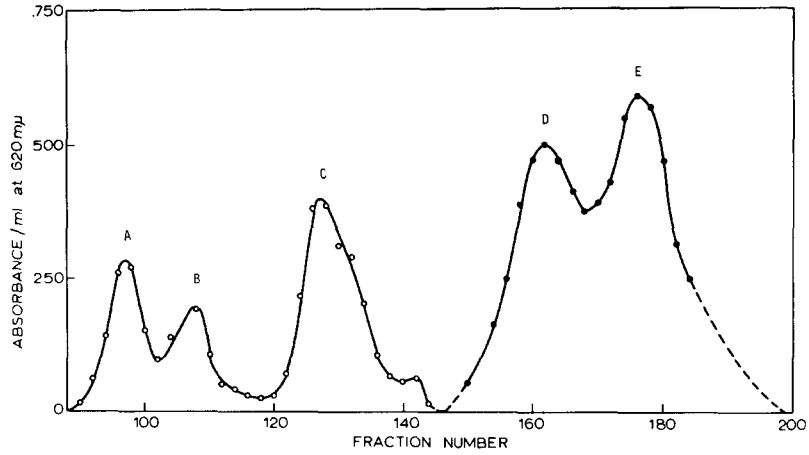

FIG. 1. Chromatogram of unsaponifiable lipids from rat skin. O—O, "fast-acting" sterols. ●—●, "slow-acting" sterols. (*A*) 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol; (*B*) 4α-methyl-Δ^{7}-cholestenol and 4α-methyl-Δ^{8}-cholestenol; (*C*) 4β-methyl-$\Delta^{8,24}$- and 4-methyl-$\Delta^{7,24}$-cholestadienols; (*D*) Cholesterol; (*E*) Desmosterol.

graphed on a 10 × 100 cm silicic acid–Celite (2:1 w/w) column (Fig. 1). Fractions 122–140 contained 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol (I)[1] and 4-methyl-$\Delta^{7,24}$-cholestadien-3β-ol (II), in accord with the observation of Clayton et al. (1). In order to determine the per cent contamination of I by II, the methyl ethers of aliquots from individual fractions 123, 127, 135, and 137 were analyzed by GLC. As can be seen from Table 1, the per cent concentration of II increases from 14.4% in fraction 123 to 20% in fraction 137 as the more polar edge (right) of the peak is approached. In order to reduce further the amount of II, fractions 122–138 (260 ml each) were reduced in volume to 40 ml each and were applied, one by one and without external pressure, to a 4 × 200 cm column in the same order in which they were eluted from the previous 10 × 100 cm column. A single peak with a slight shoulder on the more polar edge emerged. The GLC analysis of small aliquots of fractions from this peak showed that the over-all contamination of I by II was now reduced from 16 to about 6%. The fractions comprising the first three-fourths of the peak were pooled and were evaporated to dryness under nitrogen. The residue (229.5 mg) was treated with digitonin according to the method of Sperry (7). The free sterols were liberated by treatment of the digitonide with freshly dried pyridine, and ethyl ether was added to precipitate the digitonin. The weight of the free sterols thus obtained was 125 mg. Crystallization from anhydrous methanol yielded 72.2 mg of extremely fine white crystals, mp 125°–126°C. Two further crystallizations yielded 62 mg of material and raised the melting point to a constant 134.5°–136°C. The mother liquors were combined, and the sterols were crystallized three times to a constant melting point (135.5°–136.2°C), (43.7 mg). Both crops of sterols were combined to yield 105 mg of I.

RESULTS AND DISCUSSION

As judged by GLC, the purity of compound I was 95.5%, the remaining 4.5% being made up of II. The retention time, relative to 5α-cholestane, of the methyl ether of I was 5.67, consistent with the predicted retention time of 5.69 for the methyl ether of a 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol. Elemental analysis for C and H of I indicated a

TABLE 1 PERCENTAGE CONTAMINATION OF I BY II*

Fraction No.	Composition†		Resolution‡
	I	II	
	%	%	
123	85.6	14.4	1.24
127	86.6	13.4	1.23
135	84.3	15.7	1.24
137	80.0	20.0	1.27

* See Text for identification of I and II.

† Calculated by triangulation. These values are for the material that emerged from the GLC column.

‡ Signifies the degree of separation achieved on GLC. The resolution is the ratio of retention time of II to that of I.

[1] Names of sterols used are: 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol, 4β-methyl-5α-cholesta-8,24-dien-3β-ol; 4-methyl-$\Delta^{7,24}$-cholestadien-3β-ol, 4-methyl-5α-cholesta-7,24-dien-3β-ol; 4-methyl-$\Delta^{8,24}$-cholestadien-3-one, 4β-methyl-5α-cholesta-8,24-dien-3-one; Δ^{7}-cholesten-3-one, 5α-cholest-7-en-3-one; $\Delta^{8(14)}$-ergosten-3-one, 5α-ergost-8(14)-en-3-one; 4α-methyl-Δ^{8}-cholestenol, 4α-methyl-5α-cholest-8-en-3β-ol; Δ^{8}-cholestenol, 5α-cholest-8-en-3β-ol; $\Delta^{8,24}$-cholestadienol, 5α-cholesta-8,24-dien-3β-ol (zymosterol); 4α-methyl-Δ^{7}-cholesten-3β-ol, 4α-methyl-5α-cholest-7-en-3β-ol (lophenol); $\Delta^{8(14)}$-cholesten-3β-ol, 5α-cholest-8(14)-en-3β-ol; Δ^{7}-cholestenol, 5α-cholest-7-en-3β-ol; Δ^{5}-cholestenol, cholest-5-en-3β-ol (cholesterol); Δ^{24}-cholesten-3β-ol, 5α-cholest-24-en-3β-ol; $\Delta^{5,24}$-cholestadien-3β-ol, cholesta-5,24-dien-3β-ol (desmosterol); $\Delta^{7,24}$-cholestadien-3β-ol, 5α-cholesta-7,24-dien-3β-ol; 4-methyl-Δ^{8}-cholesten-3β-ol, 4β-methyl-5α-cholest-8-en-3β-ol.

[2] Frantz, I. D. Jr., and G. J. Schroepfer. Unpublished data.

[3] Bhacca, N. S., and A. Sanghvi. Unpublished data.

C-28 compound with molecular weight of 398.

Analysis:	$C_{28}H_{46}O$; calculated:	C, 84.35; H, 11.63
	$C_{28}H_{48}O$; calculated:	C, 83.93; H, 12.07
	I; found :	C, 83.58; H, 11.60

The molecular weight was confirmed by the molecular ion peak m/e 398 in the mass spectrum of I and the m/e 396 molecular ion peak in the mass spectrum of 4-methyl-$\Delta^{8,24}$-cholestadien-3-one (IV) derived from a Jones oxidation (8, 9) of I in 8 N chromium trioxide–sulfuric acid solution. A further analysis of the mass spectra of I and IV revealed that the "extra" methyl group was present in the tetracyclic skeleton and not in the side chain attached to ring D since characteristic fragment peaks were observed at m/e 245 and m/e 243 in the mass spectra of I and IV, respectively. Appearance of the above two fragments is consonant with the splitting off of a C_8H_{15} side chain plus 42 m.u. (M-[C_8H_{15} + 42]), one of the most commonly observed features in the mass spectra of C-17 substituted steroids (10). A fragment peak at m/e 313 (M-83) in the mass spectrum of IV was also in accord with elision of ring A containing the extra methyl group at position C-1 or C-2 or C-4.

A brief summary of the ORD data which is consistent with the C-4β assignment to the substituent methyl group in I is as follows (see reference 2 for details). The ORD curve of IV in methanol showed a positive Cotton effect (11) characteristic of the 3-keto-5α-steroids, and 3-keto steroids with a 5β-configuration are commonly known to exhibit a negative Cotton effect (12). The presence of a double bond in ring B is known not to alter the positive nature of the Cotton effect except to cause changes in the amplitude of the dispersion curve of the 3-keto steroids belonging to the 5α-cholestane series (13). The molecular amplitude (14) a $\{a = ([\phi]_1 - [\phi]_2)/100$, where $[\phi]_1$ and $[\phi]_2$ are the extremum molecular rotation values of the Cotton effect at the longer and shorter wavelengths, respectively$\}$ of IV was +19. This value was compared with a = +63, +73, +54, and +11 for 2α-, 2β-, 4α-, and 4β-methyl-5α-cholestan-3-ones, respectively. The value of +19 was closest to the +11 value for the 4β-methyl-5α-cholestan-3-one. Further, Djerassi, Halpern, Halpern, and Riniker (13) have observed that the molecular amplitude for Δ^8-cholesten-3-one is +48, and from an examination of ORD data for a number of 3-keto-5α-steroids, Allinger and DaRooge (15) have suggested that a C-2 or C-4 axial methyl contributes ±31 to the molecular amplitudes of these compounds, with the sign as predicted by the octant rule (16). Therefore, since the C-4 axial methyl lies in the negative octant, subtraction of 31 from 48 gives an a value of +17 for IV, which compares well with experimental value of +19. This observation strongly suggested that the substituent methyl group in IV had an axial configuration. Conclusive evidence for the axial nature of the C-4 methyl was obtained when IV was boiled under reflux for 2 hr with ethanol–sulfuric acid solution to induce isomerization of any 4β-methyl group to the energetically more favorable 4α-configuration (17), and the ORD curve of the resultant product showed an a value of +56. This value compares with a = +54 for 4α-methyl-5α-cholestan-3-one.

The Δ^8-Bond

The molecular amplitudes computed from the ORD curves of Δ^7-cholesten-3-one and $\Delta^{8(14)}$-ergosten-3-one are +63 and +72, respectively (13). Upon subtracting 31 as the contribution of the C-4 axial methyl group (see above), the a values one derives for the above two structural alternatives for the location of the nuclear double bond are a = +32 and a = +41, respectively. These values are considerably higher than the observed value of a = +19 for IV. The presence of the $\Delta^{8(14)}$-double bond in I is further discounted on the basis of the specific rotation and the NMR data considered below.

When treated with Liebermann-Burchard reagent, I gave an immediate blue color which reached a maximum at 620 nm 6 min after the addition of the reagent. Similar colorimetric behavior has also been observed with 4α-methyl-Δ^8-cholestenol (18), Δ^8-cholestenol (19), and $\Delta^{8,24}$-cholestadienol[2], and thus appears to be characteristic of a Δ^8-bond. In contrast, 4α-methyl-Δ^7 cholesten-3β-ol attains maximum color intensity in 1.5 min after the addition of the reagent (20). The presence of $\Delta^{5,7}$-diene was contraindicated by the failure of I to exhibit the ultraviolet absorption characteristic of the $\Delta^{5,7}$-conjugated diene system (21).

The specific rotation, $(\alpha)_D$ of I, determined at ambient temperature (ca 25°C) in chloroform, was +50.6 (c, 1.70). On the other hand, the $(\alpha)_D$ for $\Delta^{8(14)}$-cholesten-3β-ol was reported by Frantz, Davidson, Dulit, and Mobberley (22) and by Lee, Lusky, and Schroepfer (23) to be +20 and +24.4, respectively. The $(\alpha)_D$ value of I should be compared with that of zymosterol ($\Delta^{8,24}$-cholestadien-3β-ol) and with that of Δ^8-cholestenol. Both these compounds have been reported to have an $(\alpha)_D$ of +50 (24–26). It is evident from the above discussion and also from the observations by others (6, 27) that the contribution of the Δ^{24}-bond to the specific rotation, if any, is of negligible importance because of its remoteness from the rest of the molecule. Similarly, the alkyl substitution at C-4 does not seem to affect the specific rotation, since the $(\alpha)_D$ for 4α-methyl-Δ^8-cholestenol is +55 (18). Lophenol (4α-methyl-Δ^7-cholestenol) had an $(\alpha)_D$ of +5 (28), and three values of $(\alpha)_D$ have been reported for Δ^7-cholestenol: +3.9 (29), +5.65 (30), and +6.5 (31). Thus, the specific rotation of +50.6 is compatible with the presence of Δ^8-bond in I.

TABLE 2 PREDICTED CHEMICAL SHIFTS OF 19-H AND 18-H IN 4β-METHYL-5α-CHOLESTAN-3β-OL IN THE PRESENCE OF DOUBLE BONDS AT DIFFERENT NUCLEAR LOCATIONS*

	19-H		18-H
4β-CH_3-5α-cholestan-3β-ol	0.878		0.650
$\Delta^{8(9)}$	1.003	δ, *p.p.m.*	0.567
$\Delta^{8(14)}$	0.761		0.825
Δ^{7}	1.153		0.658
Δ^{5}	0.853		0.642
$\Delta^{5,7}$	1.020		0.625

* An example of these calculations is as follows:

	19-H		18-H
5α,14α-androstane	0.792	*p.p.m.*	0.692
3β-OH	+0.033		+0.008
Δ^{8}-	+0.125		−0.083
C_8H_{17}-C_8H_{15}	−0.017		−0.050
4β-CH_3	+0.070		−0.000
calc.	δ 1.003		δ 0.567
obs. (I)	0.957		0.595
obs. (III)	0.975		0.608

Additional evidence for C-8(9) unsaturation in I comes from a consideration of the NMR data. It is now recognized that the nature and the orientation of substituents in a steroid skeleton affect the chemical shifts of the angular C-18 and C-19 methyl protons and that this effect is additive in an algebraic sense (32–34). From a recognition of this phenomenon and from the published data (35), it is possible to calculate the resonance frequencies of C-18 and C-19 methyl protons of a given sterol molecule with double bonds at different nuclear locations. These predicted chemical shifts are shown in Table 2. These calculations indicate that the observed chemical shifts for the C-18 and C-19 methyls of I and III (0.595, 0.957; 0.608, 0.975; respectively, Figs. 2 and 4) are most compatible with the double bond in I occupying the 8(9) position.

Some comment must be made concerning the contribution of the C-4β-methyl group to the chemical shift of the C-19 methyl protons. The contribution of +0.070 p.p.m. (Table 2) for 4β-CH_3 to the chemical shift of C-19 methyl is from a report by Cohen and Rock (36), in which are listed the effects of substituents on 5α, 14α-H sterols that contain only Δ^{4}- or Δ^{5}- unsaturation. The Dreiding models show that insertion of the Δ^{8}-bond introduces conformational distortion in ring B which, in turn, also modifies the distance and the relative positions of the two synaxial methyls at C-4 and C-10. In this event, the contribution of the substituent C-4 methyl to the chemical shift of the C-19 methyl is altered, and the calculations based on the additivity principle are no longer accurate. Although an uncertainty of approximately 7 cps would not critically affect the present case, conformational considerations require that conclusions regarding the substituent effects must be drawn with caution.

The Δ^{24}-Bond

Fig. 3 shows an IR spectrum of I. Jones and Cole (37) have observed that in the absence of a Δ^{24}- or a Δ^{25}-bond the terminal gem-dimethyl group of the steroidal side chain gives rise to an absorption band at 1368 cm^{-1}, and that a diminution in absorption at that frequency is compatible with the presence of a Δ^{24}- or Δ^{25}-bond. Accordingly, cholesterol (Δ^{5}-cholestenol) and Δ^{7}-cholestenol exhibit prominent absorption at 1368 cm^{-1}

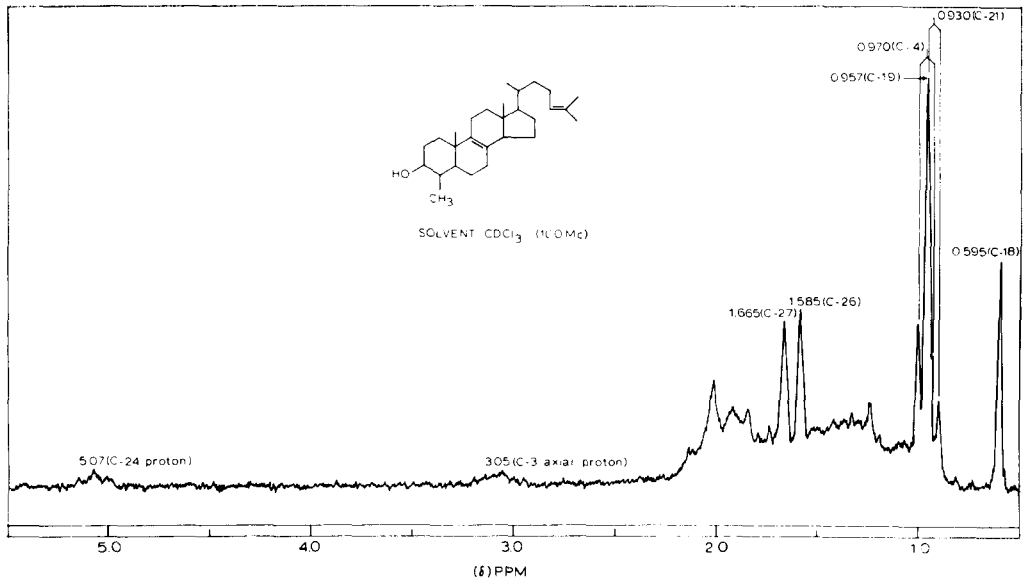

FIG. 2. NMR spectrum of 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol.

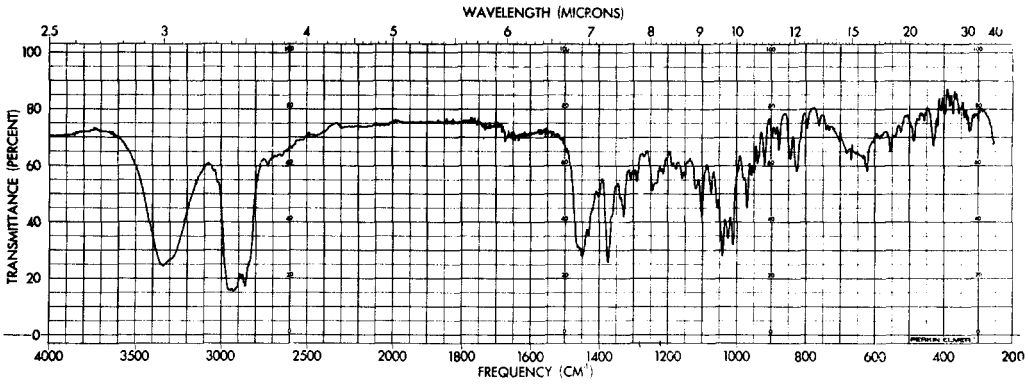

FIG. 3. Infrared spectrum of 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol.

whereas their C-24(25) unsaturated analogues show a much diminished absorption at the same frequency (6). An examination of Fig. 3 reveals the presence of a small absorption shoulder at 1368 cm^{-1}, in accord with the above observations and compatible with the presence of a Δ^{24}- or Δ^{25}-bond in I. Absence of an absorption band at 887 cm^{-1} eliminates the Δ^{25}-bond as an alternative possibility (38). Recently published data of Scallen and Krueger (39) are in agreement with these points.

NMR data for several sterols which contain a Δ^{24}-bond in the side chain indicate that C-26 and C-27 methyl protons give two distinct signals in the region 1.57 p.p.m. and 1.67 p.p.m., respectively. The following examples illustrate this point: Δ^{24}-cholesten-3β-ol, 1.58, 1.67 p.p.m. (40); $\Delta^{5,24}$-cholestadien-3β-ol, 1.57, 1.65 p.p.m. (41); $\Delta^{7,24}$-cholestadien-3β-ol, 1.58, 1.67 p.p.m. (6). In Fig. 2, the two resonances at 1.585 p.p.m. and 1.665 p.p.m. thus represent the C-26 and C-27 isopropylidene methyls. According to Bates and Gale (42), the upfield resonance at 1.585 p.p.m. is due to the C-26 methyl trans to the C-24 hydrogen, and the downfield signal at 1.665 p.p.m. is then due to the C-27 methyl group. The long C-24 proton directly attached to the doubly bonded carbon appears at 5.07 p.p.m., and this is consistent with the observations we have made with a series of C-24(25) unsaturated steroids.[3]

Reduction of the Δ^{24}-Bond

5 ml of purified dioxane (43) were added to 10.39 mg of I which was then hydrogenated over freshly prepared Raney nickel catalyst (44). The product was purified by passage through a silicic acid-Celite column and it was recrystallized from anhydrous methanol-acetone to a constant m.p. 134–134.5°C. The retention time, relative to 5α-cholestane, of the methyl ether of this material (4-methyl-Δ^{8}-cholesten-3β-ol, III) as determined by GLC was 3.9, compared to a retention time of 3.95 for a 4-methyl-Δ^{8}-sterol methyl ether as shown by Clayton et al. (1). A small peak accompanying the large one had the retention time of 4.88, which corresponds to the retention time of a 4-methyl-Δ^{7}-sterol methyl ether. This peak constituted approximately 4.5% of the total area which is compatible with the amount of II present as a contaminant in I. No material emerged from the GLC column with a retention time corresponding to that of the methyl ether of I.

The NMR data obtained for III provide further evidence of the reduction of the Δ^{24}-bond of I. Fig. 4 shows the region 0.5–1.2 p.p.m. of an expanded NMR spectrum of III. Two downfield signals at 1.585 p.p.m. and 1.665 p.p.m. due to C-26 and C-27 isopropylidiene methyls in the NMR spectrum of I (Fig. 2) have moved upfield (Fig. 4) and now occur at 0.834 p.p.m. and 0.896 p.p.m. These represent a six proton doublet centered at 0.865 p.p.m. corresponding to the two secondary methyls attached to C-25. This information is consistent with the presence of a Δ^{24}-bond in I and with its absence in III.

CONCLUSION

The evidence presented here taken in conjunction with the optical rotatory dispersion and mass spectrometric data presented elsewhere (2) permit the conclusion that the isolated sterol has the structure 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol.

Earlier (2), we have commented that the isolation of 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol had some relevance on the sequence of demethylation at C-4 along the biosynthetic pathway from lanosterol to cholesterol. We suggested that, at least in this instance, the 4α-methyl group can be removed before the 4β-methyl group. Of particular interest in this regard was the observation by Allinger and DaRooge (15) that in 4,4-dimethyl-3-keto steroids ring A is not a perfect chair, but rather that it has a "flat chair" conformation. In this form the 4α-methyl group is considerably more exposed than the 4β-methyl group, and this would facilitate its enzymatic removal. Recent work of Sharpless et al. (45) on the biological demethyla-

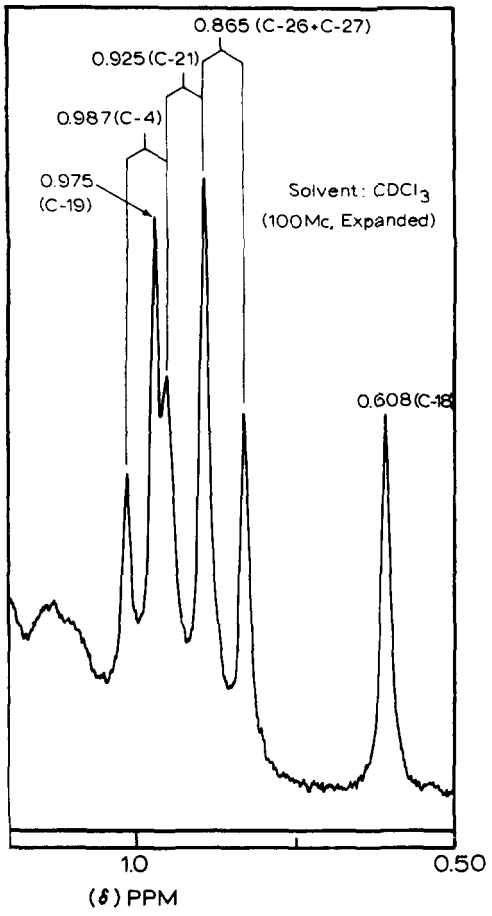

FIG. 4. NMR spectrum of 4-methyl-Δ^8-cholesten-3β-ol.

tion of 4,4-dimethyl sterols appears to confirm this point. An account of the conversion of 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol to cholesterol is the subject of another report to be published.

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