On the β Configuration of the C-4 Methyl in a 4-Methyl-$\Delta^{8,24}$-Cholestadien-3β-ol Isolated from Rat Skin*

Ajit Sanghvi,† D. Balasubramanian, and Albert Moscowitz

ABSTRACT: A new sterol has recently been isolated from the skins of triparanol-treated rats. Previous workers had tentatively assigned to it the structure 4α-methyl-$\Delta^{8,24}$-cholestadien-3β-ol.

From mass spectral data, optical rotatory dispersion data, and data from isomerization studies it is shown that the monomethyl substituent actually has the 4β configuration. This constitutes the first time that a 4β-methyl sterol has been found in mammalian tissue.

Sanghvi and Frantz (1966) have recently isolated a new monomethyl sterol from the skins of rats that were treated with the drug triparanol.[1] Clayton *et al.* (1963), who first detected the presence of this sterol in the skins of triparanol-treated rats, assigned to it the tentative structure 4α-methyl-$\Delta^{8,24}$-cholestadien-3β-ol. As described elsewhere (Sanghvi and Frantz, 1966), the assignment of the sterol as a monomethyl-$\Delta^{8,24}$-cholestadien-3β-ol has since been confirmed. However, using the techniques of mass spectrometry and optical rotatory dispersion (ORD) we have gathered compelling evidence that the methyl group at position C-4 has a β rather than an α configuration. This result is of particular significance since it represents the first time that a 4β-methyl sterol has been isolated from mammalian tissue; to the best of our knowledge, all previously reported monomethyl sterols isolated from mammalian sources (Neiderhiser and Wells, 1959); Kandutsch and Russell, 1960, Bloch, 1965) have been assigned with the C-4 methyl in the α configuration.

Methods

The mass spectra were obtained on a Hitachi RMU 6D mass spectrometer with a direct inlet system at an ionizing potential of 50 ev. Optical rotatory dispersion measurements were made at ambient temperature ($\sim$25°) with a Cary Model 60 spectropolarimeter using 1-cm cells. Spectral grade methanol was the solvent in all cases. The reproducibility of the observations was within $\pm 1 \times 10^{-3}$ degrees. The data are presented as molecular rotations [Φ], and are discussed in terms of molecular amplitudes (Djerassi and Klyne, 1962), $a = ([\Phi]_1 - [\Phi]_2)/100$, where $[\Phi]_1$ and $[\Phi]_2$ are the extremum values of the Cotton effect at the longer and shorter wavelengths, respectively.

Mass Spectrometric Results and Interpretation

Elemental analysis for C, H (Calcd: C, 84.36; H, 11.63. Found: C, 83.58; H, 11.60.) of the sterol indicates a C_{28} compound consistent with a molecular weight of 398. The molecular weight is confirmed by the molecular ion peak at *m/e* 398 in the mass spectrum of the 4-methyl-$\Delta^{8,24}$-cholestadien-3β-ol (I) (Figure 1) and the *m/e* 396 molecular ion peak in the mass spectrum (Figure 2) of the 4-methyl-$\Delta^{8,24}$-cholestadien-3-one (II) derived from a Jones oxidation of I in 8 N chromium trioxide–sulfuric acid solution (Bowden *et al.*, 1946; Djerassi *et al.*, 1956). Characteristic peaks also appear at *m/e* 245 and *m/e* 243 in the mass spectra of I and II, respectively, in accord with the splitting off of a C_8H_{15} side chain plus 42 mass units [M − (C_8H_{15} + 42)], as is commonly observed for steroids and oxygen-substituted steroids (Biemann, 1962; Budzikiewicz *et al.*, 1964); hence, the presence of these latter peaks indicates that the methyl group in question is present in the tetracyclic skeleton and not in the side chain attached to ring D. Further, the appearance of a fragment peak at *m/e* 313 [M − 83] in the mass spectrum of II is consonant with elision of the A ring containing the extra methyl group at position C-1 or C-2 or C-4.

In summary, the elemental analysis and mass spectral data, taken in conjunction with the work of Clayton *et al.* (1963) and Sanghvi and Frantz (1966), indicate that the compound in question is a monomethyl-$\Delta^{8,24}$-cholestadien-3β-ol with the substituted methyl group in the A ring at positions C-1 or C-2 or C-4. It remains

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† All inquiries including the inquiries regarding the reprints of this article should be directed to this author's present address: Department of Medicine, University of Minnesota Hospitals, Minneapolis, Minn. 55455.

[1] Abbreviation used: MER-29, 1-[4-(dimethylaminoethoxy)-phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol.

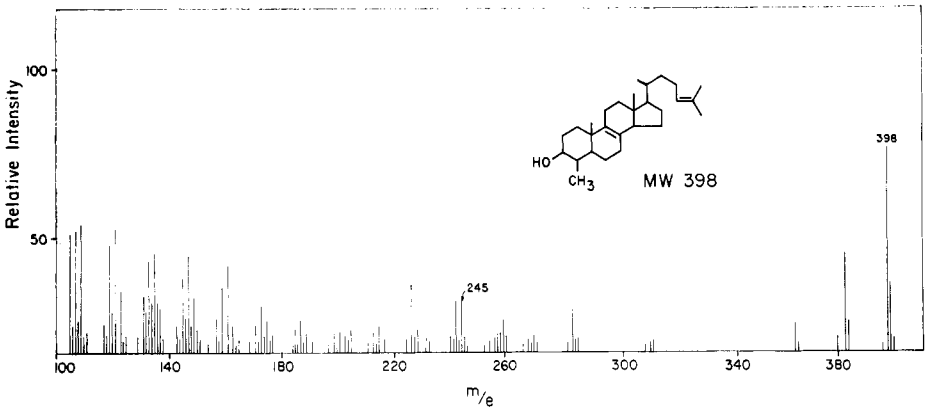

FIGURE 1: Mass spectrum of 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol.

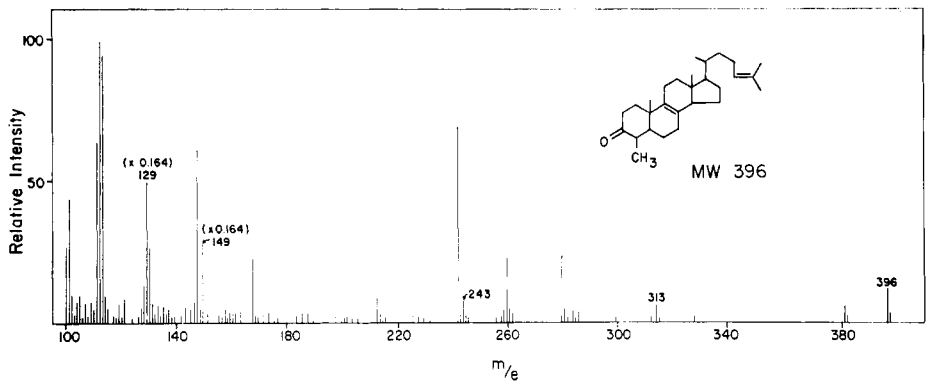

FIGURE 2: Mass spectrum of 4β-methyl-$\Delta^{8,24}$-cholestadien-3-one.

to ascertain its precise position and configuration.

ORD Results and Interpretation

In order to determine the point of attachment and stereochemistry of the methyl group in question, the (hemi)ketal technique developed by Djerassi and co-workers (1959) was employed. This method depends upon the fact that: (a) Significant conversion of a six-membered cyclic ketone to the corresponding (hemi)ketal by the addition of HCl to a methanolic solution of the ketone is reflected in a marked diminution in the magnitude of the 300-mμ Cotton effect characteristic of the carbonyl n-π^* transition. (b) The extent of (hemi)ketal formation and the concomitant reduction in the amplitude of the 300-mμ Cotton effect is critically dependent on the stereochemical environment of the C=O group. In particular, (hemi)ketal formation is severely inhibited by methyl groups attached to carbon atoms adjacent to the C=O group, or by axial methyl groups on next to nearest neighboring carbon atoms; in the latter situation, the presence of such axial methyl substituents would lead to new and unfavorable 1,3-diaxial interactions upon (hemi)ketal formation (Djerassi, 1960).

Figure 3 shows the ORD curve of II derived by Jones oxidation of I. Addition of HCl to the methanolic solution produced virtually no change in the amplitude of the 300-mμ Cotton effect, indicating a marked inhibition of (hemi)ketal formation. Therefore, in agreement with the inferences from the mass spectral data, one may conclude that the methyl group is in the A ring and, further, that it cannot be in the C-1 equatorial position. Invocation of the octant rule (Moffitt *et al.*, 1961) also excludes the C-1 axial position, since such a 1α-methyl-$\Delta^{8,24}$-cholestadien-3-one would be predicted to give rise to a negative Cotton effect. Thus, the C-1 position is eliminated as a possible point of attachment.

A comparison of the molecular amplitude (Djerassi and Klyne, 1962), *a*, of the 300-mμ Cotton effect of II with *a* values for pertinent 2α- and 2β-, and 4α- and 4β-methyl-3-keto steroids helps distinguish between the C-2 and C-4 positions. Ideally, comparisons would be made with relevant monomethyl-$\Delta^{8,24}$-cholestadienones. However, in the absence of such data, the *a* values for 2α- and 2β-, and 4α- and 4β-methyl-5α-cholestan-3-ones will be employed. Some justification can be made for this. The effect of the Δ^{24} double bond is of negligible importance because of the distance of the side chain from the C=O group.

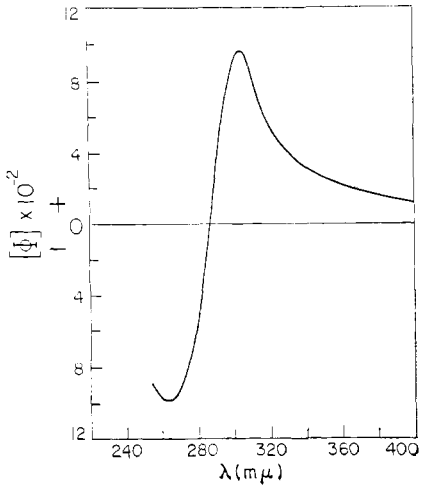

FIGURE 3: ORD curve of 4β-methyl-$\Delta^{8,24}$-cholestadien-3-one (c 0.2%, methanol).

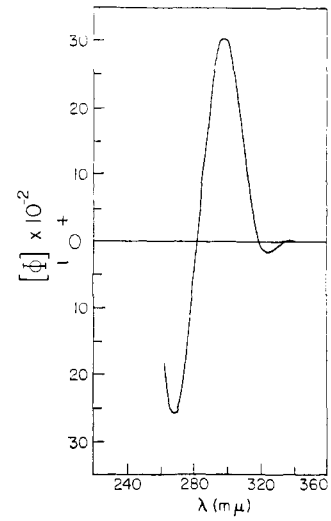

FIGURE 4: ORD curve of the 4α-methylcholestadien-3-one, obtained as a product of acid-catalyzed isomerization of 4β-methyl-$\Delta^{8,24}$-cholestadien-3-one (c 0.028%, methanol).

The effect of the Δ^8 double bond is more serious, but its contribution to the molecular amplitude in $\Delta^{8(9)}$-cholesten-3-one can be estimated from available data to introduce an uncertainty of the order of 10–15 units, and an uncertainty of this magnitude will not critically affect the considerations which follow.

The molecular amplitude of II is +19. This value is to be compared with a = +63 and +73 for 2α- and 2β-methyl-5α-cholestan-3-ones, respectively, and a = +54 and +11 for 4α- and 4β-methyl-5α-cholestan-3-ones, respectively. The value of +19 is closest to the +11 value for the 4β-methyl-5α-cholestan-3-one, but in any case, both the C-2 configurations are ruled out entirely. Moreover, from an examination of ORD data for 3-keto steroids, Allinger and DaRooge (1962) have suggested that a C-2 or C-4 axial methyl group contributes ±31 units to the molecular amplitude of these compounds, with the sign as predicted by the octant rule, and Δ^8-cholesten-3-one has a molecular amplitude of +48 (Djerassi *et al.*, 1958). Therefore, since the 4β-methyl group lies in a negative octant, upon subtracting 31 from 48, one calculates an a of +17 for II, which compares quite favorably with the experimental value of a = +19, and very strongly suggests that the substituent methyl group is C-4β. However, even more cogent evidence comes from an isomerization study of the indicated 4β-methyl to the energetically more favorable 4α configuration.

Compound I (2 mg) was oxidized with 8 N chromium trioxide–sulfuric acid solution (Jones oxidation) and the II so derived was boiled under reflux for 2 hr with 3 ml of ethanol containing 0.3 ml of 20% sulfuric acid to induce isomerization of any 4β-methyl group to the 4α configuration (Mazur and Sondheimer, 1958). Isolation with ether and purification through a small silicic acid–Celite (2:1, w/w) column yielded 0.7 mg of a 4-methylcholestadien-3-one. The compound was dissolved in 2.5 ml of spectral grade methanol, and the ORD curve was taken (Figure 4). The a value derived from the curve is +56, which compares quite well with a = +54 for 4α-methyl-5α-cholestan-3-one.

Although the Jones oxidation is too mild a treatment to cause any migration of the $\Delta^{8(9)}$ double bond, the subsequent treatment with sulfuric acid–ethanol could conceivably cause such double bond migration, and one might argue that the ORD differences between Figures 3 and 4 were the consequence of double bond migration, rather than the result of isomerization of the methyl group from the 4β to the 4α configuration. However, this possibility can be ruled out on the following grounds.

If conditions are drastic enough so that double bond migration from $\Delta^{8(9)}$ to $\Delta^{8(14)}$ were to occur, the double bond migration would not stop at $\Delta^{8(14)}$ but continue on to the $\Delta^{14(15)}$ position (Fieser and Fieser, 1959). But Djerassi *et al.* (1958) have shown that the molecular amplitudes of $\Delta^{8(9)}$-cholesten-3-one (a = +48) and $\Delta^{14(15)}$-ergosten-3-one (a = +54) are roughly the same, *i.e.*, that it makes little difference for the molecular amplitude whether the double bond occupies the $\Delta^{8(9)}$ or the $\Delta^{14(15)}$ position. It follows then that double bond migration cannot account for the large differences between the a values associated with the curves in Figures 3 and 4.

It should be stated that the methanol solution used in the measurements of the curve for Figure 4 was faintly yellow, an indication of a slight impurity, possibly an α,β-unsaturated ketone. This last conjecture is in accord with the small extremum in the vicinity of 320 mμ in Figure 4. However, the presence of a small amount of such an impurity in no way invalidates the conclusion drawn that what was principally observed

was an isomerization of a 4β-methyl to a 4α-methyl configuration.

Additional Remarks

The isolation of 4β-methyl-$\Delta^{8,24}$-cholestadien-3β-ol has some relevance for the sequence of demethylation at C-4 along the biogenetic pathway from lanosterol to cholesterol. At least in the present instance, it appears that the 4α-methyl can be removed before the 4β-methyl group. In this connection, it is perhaps pertinent to note that recent conformational studies (Allinger and DaRooge, 1962) on 4,4-dimethyl-3-keto steroids present cogent evidence for the fact that in these steroids the A ring is not in the classical chair or boat conformations. Rather, it has the form of a "flattened chair" in which the methyl groups at C-4 are neither strictly axial nor equatorial. In such a conformation, the 4α-methyl is considerably more exposed than the 4β-methyl group.

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References

Allinger, N. L., and DeRooge, M. A. (1962), *J. Am. Chem. Soc. 84*, 4561.

Biemann, K. (1962), Mass Spectrometry: Organic Chemical Applications, New York, N. Y., McGraw-Hill.

Bloch, K. (1965), *Science 150*, 19.

Bowden, K., Heilborn, I. M., Jones, E. R. H., and Weedon, B. C. L. (1946), *J. Chem. Soc.*, 39.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964), Structure Elucidation of Natural Products by Mass Spectrometry, Vol. II, San Francisco, Calif., Holden-Day.

Clayton, R. B., Nelson, A. N., and Frantz, I. D., Jr. (1963), *J. Lipid Res. 4*, 166.

Djerassi, C. (1960), Optical Rotatory Dispersion: Applications to Organic Chemistry, New York, N. Y., McGraw-Hill.

Djerassi, C., Engle, R. R., and Bowers, A. (1956), *J. Org. Chem. 21*, 1547.

Djerassi, C., Halpern, O., Halpern, V., and Riniker, B. (1958), *J. Am. Chem. Soc. 80*, 4001.

Djerassi, C., and Klyne, W. (1962), *J. Chem. Soc.*, 4929.

Djerassi, C., Mitscher, L. A., and Mitscher, B. V. (1959), *J. Am. Chem. Soc. 81*, 947.

Fieser, L. F., and Fieser, M. (1959), Steroids, New York, N. Y., Reinhold, p 261.

Kandutsch, A. A., and Russell, A. E. (1960), *J. Am. Chem. Soc. 80*, 2589.

Mazur, Y., and Sondheimer, F. (1958), *J. Am. Chem. Soc. 80*, 2489.

Moffitt, W., Woodward, R. B., Moscowitz, A., Klyne, W., and Djerassi, C. (1961), *J. Am. Chem. Soc. 83*, 4013.

Neiderhiser, D. H., and Wells, W. W. (1959), *Arch. Biochem. Biophys. 81*, 300.

Sanghvi, A., and Frantz, I. D. (1966), Ph.D. Thesis of A. Sanghri, University of Minnesota, Minneapolis, Minn.