PROOF OF STRUCTURE FOR 24β-METHYLCHOLESTEROL IN THE ALGA *COCCOMYXA ELONGATA* BY ^{1}H- AND ^{13}C-NMR AND MASS SPECTROSCOPY

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ABSTRACT.—The green alga *Coccomyxa elongata* belonging to the order Chlorosphaerales (in the older literature Tetrasporales or Volvocales) is known to contain a sterol mixture. An examination of the component in the largest amount by ^{1}H- and ^{13}C-nmr spectroscopy together with mass spectral data rigorously proved it to be 24β-methylcholesterol. In addition, our information indicated stereochemical purity. This alga, therefore, corresponds to the other green alga species investigated in that the 24-alkylsterols present have the 24β-configuration.

The chiral centers in sterols can be divided into those which are invariant and those which differ depending on the living systems in which they occur. Among the Δ^5-sterols, which appear to comprise the most common type, all of the chiral centers at ring junctions fall into the invariant category so far as is known (1, 2). Two centers in the side chain also appear to be invariant. They are at C–20 and the point (C–17) where the side chain meets the tetracyclic system (1–4). Thus, it is possible to describe all naturally occurring Δ^5-sterols as having the stereochemistry of cholesterol at C–8, C–9, C–10, C–13, C–14, C–17, and C–20. However, when C–24 is rendered chiral by the introduction of an alkyl group, variance at this position occurs. The configuration (which presumably influences function) is known to depend on taxonomy and apparently also on evolutionary status (2, 5–8). Statistically a more frequent occurrence of 24β-alkylsterols[1] is observed the lower an organism is in the evolutionary hierarchy.

Among the algae, for instance, 24β-alkylsterols are dominant but not exclusive while in angiosperms, representing the highest plants, 24α-alkylsterols (both at the 24–C_1 and 24–C_2-stages of biosynthesis) are dominant but not exclusive (2). That some sort of evolutionary process is reflected here is given credence by the sterols of the low tracheophyte, *Lycopodium*, which is intermediate between the lowest and highest of the photosynthetic plants. In *L. complanatum* one finds an unusually high amount of 24β-alkylsterol in the form of 24β-methylsterols with Δ^5- and $\Delta^{5,7}$-types of nuclear unsaturation even though the 24-ethyl component is exclusively of the α-configuration (7, 8). Consequently, in order to understand function and phylogenetics better, it is necessary to know unequivocally what the configuration at C–24 is in sterols derived from various taxonomic groups. This was first achieved spectroscopically with ^{1}H-nmr (6–13). At a field strength of at least 220 MHz, one can distinguish C–24 epimers with particular clarity. As has recently been done by Koizumi et al. (14) and Wright et al. (15), we have now extended the methodology to ^{13}C-nmr. The purpose of this paper is to describe

[1]See references 1 and 2 for a discussion of the α,β-convention of nomenclature in the side chain. It is preferable to the R,S-notation because nomenclature inversions occur with the latter even though absolute configurations may not change. The common 24β-alkylsterols without a Δ^{22}-bond are designated 24(S), while they are 24(R) if a Δ^{22}-bond is present.

the application of these two techniques to the problem with the sterol of a green alga.

The alga studied (*Coccomyxa elongata*) is in the division Chlorophyta, class Chlorophyceae, and has recently been placed in the order Chlorosphaerales (16). It is the only species in the order which has been examined for sterols, and a mixture was found (16). The component in highest amount (48%) was thought (16) to be 24β-methylcholesterol based on unpublished nonspectral criteria (mp and glc). We have now determined the spectra of this material, and the results prove rigidly that the original assignment was correct. In addition, our spectral analysis showed no indication of a minor diastereo-isomeric component with the opposite configuration. This purity of configuration contrasts sharply with the situation in higher plants where mixtures of 24α- and 24β-methylsterols (but not of 24-ethylsterols) are common (2, 6, 7).

EXPERIMENTAL

The sample of 24-methylcholesterol (as the acetate) was kindly provided by Dr. G. W. Patterson who had separated it chromatographically (16) from the other sterols of *Coccomyxa elongata*. Mass spectra were obtained by electron impact ionization with a direct inlet on a Hitachi-Perkin-Elmer RMU-6E spectrometer at 70 electron volts and 150°. The ^{13}C-nmr spectra were obtained in $CDCl_3$ on a Varian CFT-20 spectrometer, and ^{1}H-nmr was similarly carried out in $CDCl_3$ but a 220 MHz on a Varian instrument in a commercial arrangement with Morgan-Schaffer of Montreal, Canada.

RESULTS AND DISCUSSION

The ^{1}H- and ^{13}C-nmr spectra used to determine the structure of the alga sterol constitute nearly but not completely a "finger-print" of the whole sterol. As will be documented elsewhere, upon lengthening of the side chain of a sterol, a point is reached at which further additions of C-atoms produces no observable change in the ^{1}H-nmr spectra and only marginal changes in the ^{13}C-spectra. For this reason, prior to our nmr-analysis of the alga material, we examined its mass spectrum to be sure we were dealing with a compound of 28 carbon atoms. A fragmentogram was obtained at 150° at which temperature loss of acetic acid was complete (consistent with the presence of a Δ^5-bond) and no M^+ was observable. The base peak (M^+-HOAc) was *m/e* 382 equivalent to a molecular weight of 442 for the 3β-acetate and 400 for the corresponding 3β-alcohol. 24β-Methylcholesterol has a molecular weight of 400. The methyl group was located in the side chain by fragments, *e.g.*, M^+-HOAc-side chain at *m/e* 255, which retained only the C-atoms of the tetracyclic nucleus. Since these fragments were identical to those obtained from cholesterol, the additional methyl group must have been in the side chain.

In order to have authentic spectra to compare with those of the alga sterol, we acquired standard samples of the epimeric Δ^5-24-methylsterols. 24β-Methylcholesterol was prepared from ergosterol as described in the literature (17). We recently demonstrated (4) that in ergosterol the methyl group at C-24 has the β-configuration, as has often been presumed but not previously proven on a rigorous basis. An authentic sample of the epimer, 24α-methylcholesterol, was obtained from Applied Science Laboratories, Incorporated of State College, Pennsylvania, and this material is known from our earlier work (6) to be a pure entity with the assigned structure. The substance was isolated commercially from the sterol mixture of a plant. The ^{1}H-nmr spectra at 220 MHz of the two epimers (24α- and 24β-methylcholesterol) are distinctly different as shown in fig. 1 (cf. also ref. 6). Similarly, the ^{13}C-nmr spectra are quite different as shown in fig. 2.

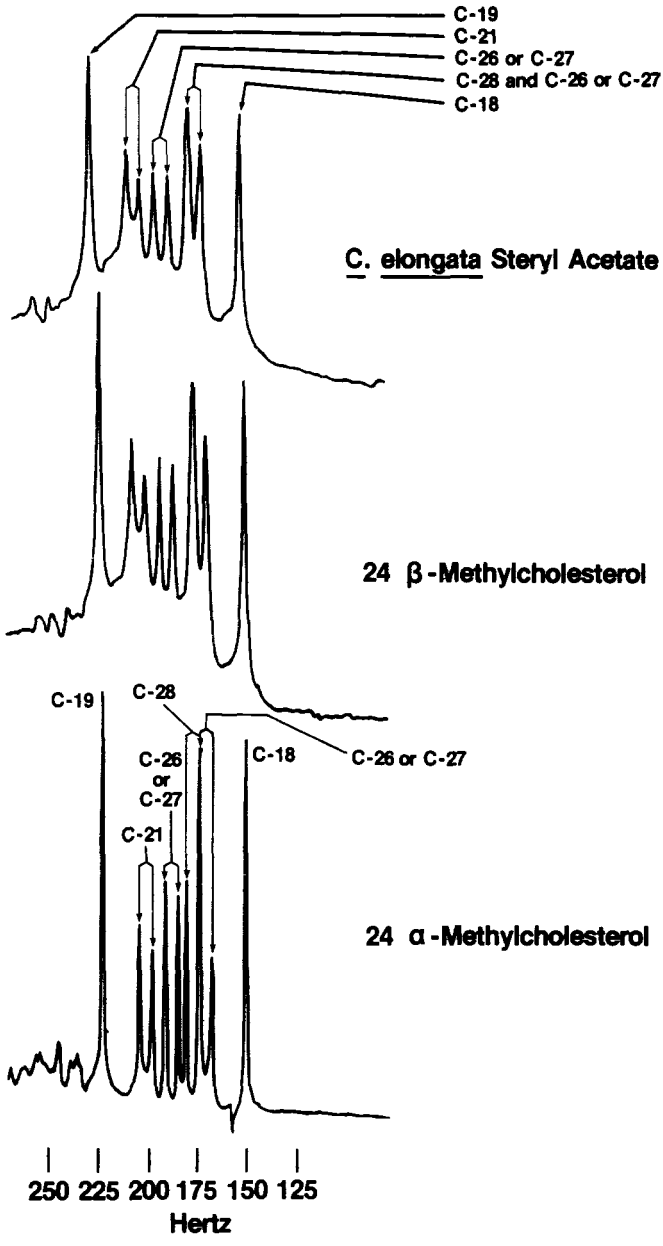

FIG. 1. The ^{1}H-nmr spectra of authentic samples of the epimeric 24-methylcholesterols and of the steryl acetate derived from *C. elongata*. Due to the proximity of the acetoxy group to C-19 in the latter case, C-19 is shifted compared to the free alcohol.

In both figures the spectra are limited to the upfield range where the resonances are found for the saturated carbon atoms which are diagnostic for configurational analysis. These two sets of reference spectra were used for the final determination

of the structure of the alga sterol which became available to us as a gift in limited quantity (a few mg) as the acetate. In order to preserve material, it was not hydrolyzed.

In the ^{1}H-nmr spectra the epimeric 24-methylcholesterols can be distinguished by small, but definite, shifts in the doublets for the protons on C–21 and C–28. With the α-epimer the position for the signal for C–21 is 2 Hz smaller and for C–28 four Hz larger than with the β-epimer. Examination of the spectra revealed the C–21 doublet of the alga sample was at the position for the β-epimer. In fig. 1 the shift for C–28 can be very clearly seen since the left-hand branch of

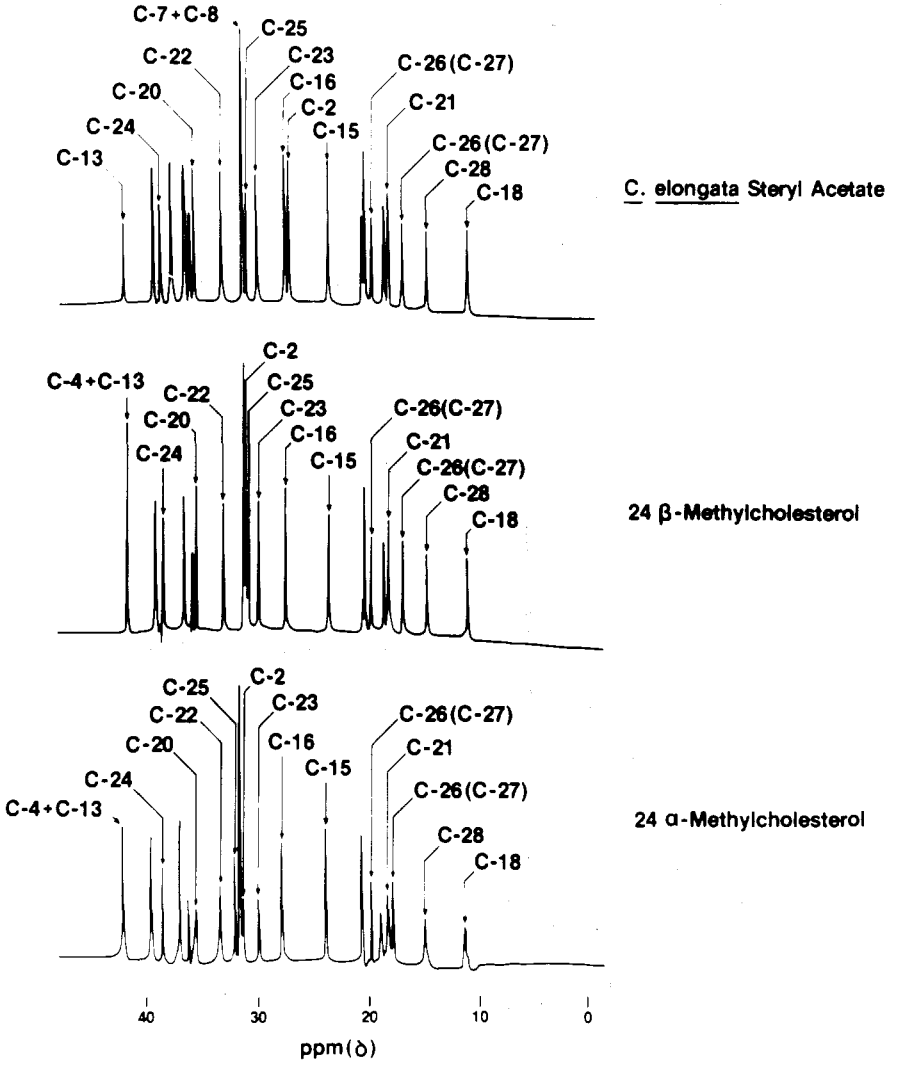

FIG. 2. The ^{13}C-nmr spectra of authentic samples of the epimeric 24-methylcholesterols and of the steryl acetate derived from *C. elongata*. See table 1 for the influence of the acetoxy group on resonances of C-atoms near C–3 and for numerical values for the positions of the peaks including those not shown here.

C–28 in the α-epimer appears at a position where there is a minimum in the spectrum of the β-epimer. This phenomenon also permits one to establish with some precision the relative amounts of the two epimers in a sample. As will be seen from fig. 1, only the β-epimer was found in the sterol of *C. elongata*.

In the ^{13}C-nmr spectra, differences between the two epimers were found to arise in the signals corresponding to C–20, C–21, C–23, C–24, C–25, C–26, and C–27 at levels varying from slight to substantial (fig. 2 and table 1). The most

TABLE 1. The ^{13}C-nmr spectra of 24α- and 24β-methylcholesterol.

Position	Value in ppm		
	C. elongata Sterol[a]	24β-Sterol	24α-Sterol
1	37.1 (37.3)	37.5	37.4
2	27.9 (31.6)	31.9	31.8
3	74.0 (71.7)	72.0	71.8
4	38.2 (42.3)	42.6	42.4
5	139.8 (140.9)	141.0	140.9
6	122.7 (121.6)	121.9	121.7
7	32.0	32.2	32.0
8	32.0	32.2	32.0
9	50.2	50.4	50.3
10	36.7	36.8	36.6
11	21.1	21.3	21.2
12	39.8	40.0	39.9
13	42.4	42.6	42.4
14	56.8	57.0	56.9
15	24.3	24.5	24.4
16	28.2	28.4	28.3
17	56.1	56.3	56.3
18	11.9	12.1	11.9
19	19.3	19.6	19.4
20	36.2	36.4	36.0
21	18.9	19.1	18.8
22	33.8	34.0	33.8
23	30.7	30.9	30.4
24	39.2	39.3	39.0
25	31.6	31.7	32.5
26			
27	17.7 & 20.5	17.9 & 20.7	18.3 & 20.2
28	15.5	15.7	15.5
CH_3 of CH_3CO	21.4		

[a]When the spectra of cholesterol and cholesteryl acetate were compared, we found the acetate gave values at C-1, C-2, C-3, C-4, C-5, and C-6 which were, respectively, +0.2, +3.7, −2.3, +4.1, +1.1, and −1.1 ppm different from the free alcohol. We therefore corrected the values for these carbon atoms in the *C. elongata* acetate so that the information could be compared with the reference spectra of free alcohols. The calculated values (corresponding to the alga free alcohol) are given in parentheses. Differences of about ±0.2 ppm are within experimental error.

extensive difference was found at C–25 which shows a chemical shift nearly one full ppm further downfield in the α- compared to the β-epimer. As will be seen numerically from table 1, the C–25 resonance of the alga sample had the value corresponding to the β-epimer. This can also be seen visually in fig. 2. The peak for C–25 is to the left of the one for C–7 and C–8 in the authentic α-epimer

and to the right of the peak for C–7 and C–8 in the authentic 24β-methylcholesterol. The peak for C–7 and C–8 can be readily distinguished by the fact that it is the most intense one in the various spectra. Since the peak for C–2 is moved away from the peak for C–7 and C–8 in the steryl acetate, C–25 is easily identified visually in the alga sample. The peak will be seen to lie just to the right of the one for C–7 and C–8, and no peak at all is to the left. This not only proves the configuration, but also shows that only a single epimer is present.

The spectral data also prove the configuration at positions other than C–24, for example, at C–20. We have previously shown (3) that in sterols with a 20β-H-atom, *e.g.*, 20-isocholesterol, there is an upfield shift in the doublet for C–21 of 0.1 ppm in the ^{1}H-nmr spectrum. At 220 MHz this amounts to 22 Hz which is about ten times the shift in this doublet occurring by virtue of inversion at C–24. It is this quantitative difference which permits the two configurational effects to be readily distinguished. Thus, 20-iso-24β-methylcholesterol should display a doublet for C–21 at 1.02 ppm instead of at 0.92 ppm, as observed with the alga sample and the reference 24β-methylcholesterol. In unpublished work from this laboratory, we have also shown inversion of the configuration at C–20 in the cholesterol case causes significant changes in the ^{13}C-spectrum. Due to exact correspondence in the ^{13}C-spectra of the alga sample and that of authentic 24β-methylcholesterol, the ^{13}C-data verify all other configurations as being the same as in the reference sample, since inversions in the nucleus would be expected to have even more profound effects than in the side chain due to the greater rigidity and chiral character of the ring system. For a discussion of the effect of stereochemical changes in the nucleus, see the article by Smith (18). In view of the fact that the spectra are also dependent on the positions of the hydroxyl group and double bond, the data presented here constitute a complete proof of structure for the sterol of *C. elongata*.

It is perhaps also worth mentioning that our numerical values for the ^{13}C-peaks of the epimers correspond within experimental error (0.2 ppm) with those for the synthetic samples (14). The assignments of peaks to the various C-atoms which we made, incidentally, were based on comparison with the spectra of cholesterol and other sterols taking into consideration relaxation times which will be published separately. Our assignments agree with both those of Koizumi *et al.* (14) and those of Wright *et al.* (15).

Sterols with a 24-ethyl group are common in green algae. Their unsaturation usually is of four types: Δ^5-, Δ^7, $\Delta^{5,22-\text{trans}}$, or $\Delta^{7,22-\text{trans}}$. In previous work (6, 7), a representative of each of these, derived from *Chlorella ellipsoidea* and *C. emersonii*, was examined spectrally by ^{1}H-nmr. Each was found to be in the 24β-ethyl series and devoid of contamination from the α-epimer. Since the 24-methylsterol examined in the present investigation was also of the 24β-configuration, there appears to be a strict configurational uniformity in green alga sterols at the 24-C_1- and 24-C_2-levels of biosynthesis in contrast to the situation in higher plants where both epimers of the 24-C_1-sterols can exist in the presence of a single epimer of 24-C_2-sterol (7).

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