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NMR SPECTRA OF C-24 ISOMERIC STEROLS

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Abstract—Cholesterol and four pairs of C-24 isomeric sterols, campesterol-22,23-dihydrobrassicasterol, α-spinasterol-chondrillasterol, stigmasterol-poriferasterol, and sitosterol-22,23-dihydroporiferasterol were studied by NMR spectroscopy and their spectra are presented. The NMR spectra of three of the pairs of isomeric sterols recorded at 100 MHz could be differentiated from each other, although at 60 MHz only the spectra of campesterol (24α-methylcholesterol) and 22,23-dihydrobrassicasterol (24β-methylcholesterol) showed differences. Sitosterol and 22,23-dihydroporiferasterol, the pair of sterols that showed no differences in their NMR spectra are readily differentiated by the physical properties of their acetates. The practical application of NMR spectroscopy to several problems concerning the C-24 isomeric sterols is demonstrated.

INTRODUCTION

INSTRUMENTS and analytical techniques are now readily available for the separation, purification, and identification of sterols of complex mixtures from plants and animals including insects. Thus, it has been possible to study the metabolism of plant sterols in insects as well as the processes involved in plant sterol biosynthesis. However, in studies concerned with the mechanism of introduction of alkyl groups at C-24 of sterols in plants, it has not always been possible to establish or relate the orientation of the alkyl substituent to that of a sterol of known stereochemistry at this position. This has been especially true where plants for specific alkylation studies have been selected because they satisfy certain experimental requirements and because they rapidly and efficiently biosynthesize a specific C-24 substituted sterol of known structure, though of unknown absolute configuration at C-24. As a result, these sterols are referred to as 24ξ-derivatives of ergostene or stigmastene. For these sterols where the structure has been established except for the stereochemistry at C-24, specific rotations have been used in assigning the configuration at this position. However, the differences in specific rotations are quite often so small that any assignment in configuration might be considered tenuous. IR and MS as well as GLC analyses at present do not differentiate these C-24 isomeric sterols. The one tool presently available with perhaps some potential in distinguishing these C-24 isomers is the NMR spectrometer.

✠ Deceased 28 December 1970.

The NMR spectra of steroids are generally complex because of the presence of a large number of aliphatic and alicyclic methylene groups. Nevertheless, Shoolery and Rogers[1] in their classical study of the NMR spectra of steroids showed that the three proton signals of the methyl groups due to the equivalence of the protons were the most pronounced and sharpest peak in the spectra and were above the background of methylene and methine protons in the region of 0·5–1·5δ. They showed that the substituents as well as stereochemical and conformational changes can have a pronounced effect on the chemical shift of these methyl groups, and in so doing demonstrated the practical application of NMR spectroscopy to structural problems of steroids. Slomp and MacKellar[2] demonstrated that even though the spectrum of steroids with methyl or ethyl groups in their side chains appeared cluttered, it was possible to identify each methyl or ethyl substituent of the steroids. Thus, it seems plausible that NMR spectroscopy could have some value in distinguishing C-24 isomeric sterols, if not through interpretative analyses of spectra perhaps by comparative analyses with reference spectra.

The availability of several pairs of C-24 isomeric sterols has permitted us to examine these sterols by NMR spectroscopy. In this paper we show that NMR spectroscopy can be used in the assignment of the configuration at C-24 for several pairs of sterols that are different at C-24. Although the assignments cannot be made directly from interpretation of spectra, the recorded NMR spectra of these sterols of known configuration at C-24 serve as reference spectra from which the stereochemistry at C-24 of an unknown sterol can be established simply by a comparative examination of NMR spectrum of a known sterol with that of the unknown. The practical application is also demonstrated.

RESULTS AND DISCUSSION

Campesterol (24α-Methyl) and 22,23-Dihydrobrassicasterol (24β-Methyl)**

Of the pairs of C-24 isomeric sterols examined by NMR spectroscopy, the pair dihydrobrassicasterol and campesterol showed the greatest differences in their spectra. Even the spectrum of campesterol (spectrum 5) and dihydrobrassicasterol (spectrum 6) recorded at 60 MHz and sweep width of 250 Hz showed a different pattern and with a noticeable difference in the position of two of the peaks in each of the respective spectra, whereas all other peaks resulting from methyl resonances appeared at a similar position in the spectrum of each of these sterols. The peaks at 45·2 and 51·5 Hz in the spectrum of campesterol, arising from the spin–spin splitting of the secondary 28-methyl protons and part of the doublet from the splitting of the 21-methyl, appeared at higher field (44·3 and 50·7 Hz) in the spectrum of 22,23-dihydrobrassicasterol. The NMR spectrum 7 of a 1:1 mixture of these two sterols shows broad bands at 44·7 and 51·5 Hz, thus confirming that the observed differences in the spectra of 22,23-dihydrobrassicasterol and campesterol were real and not caused by instrumentation. The 60 MHz spectra at 100 Hz sweep width, spectra 8 and 9 of campesterol and 22,23-dihydrobrassicasterol, respectively, show similar results, and the

* We have elected to use the symbols α and β instead of R and S since the former prefixes are commonly used now with an absolute connotation, and are well understood. However, in keeping with the recommendations of the IUPAC/IUB 1967 *Revised Tentative Rules for Steroid Nomenclature* (*Biochem. J.* **113,** 5 (1969), campesterol, sitosterol, brassicasterol, poriferasterol, and chondrillasterol have the 24R-configuration and 22,23-dihydrobrassicasterol, 22-23-dihydroporiferasterol, stigmasterol and α-spinasterol have the 24S-configuration.

[1] J. N. SHOOLERY and M. T. ROGERS, *J. Am. Chem. Soc.* **80,** 5121 (1958).
[2] G. SLOMP and F. A. MACKELLAR, *J. Am. Chem. Soc.* **84,** 204 (1962).

spectrum 10 of the mixture clearly show the contributive effects at each position where the NMR spectra of the two sterols differed (44·6–45·5 Hz and 51·0–51·7 Hz).

From the observed differences in NMR spectra of campesterol and dihydrobrassicasterol recorded at 60 MHz, one could conclude that the doublet (45·2 and 51·5 Hz) for the 28-methyl of campesterol (24α-methyl) appears at lower field than the corresponding 28-methyl of 22,23-dihydrobrassicasterol (24β-methyl). However, while this statement appears to be true, the NMR spectra of these two sterols recorded at 100 MHz and sweep widths of 100 and 250 Hz, could be interpreted differently. In the 100 MHz spectra of campesterol (spectrum 13) and 22,23-dihydrobrassicasterol (spectrum 14), the resonances of the 28-methyls are in similar positions, though the spectra are very different. The differences in the spectra of these two C-24 isomers are most likely caused by the stereochemistry at C-24.

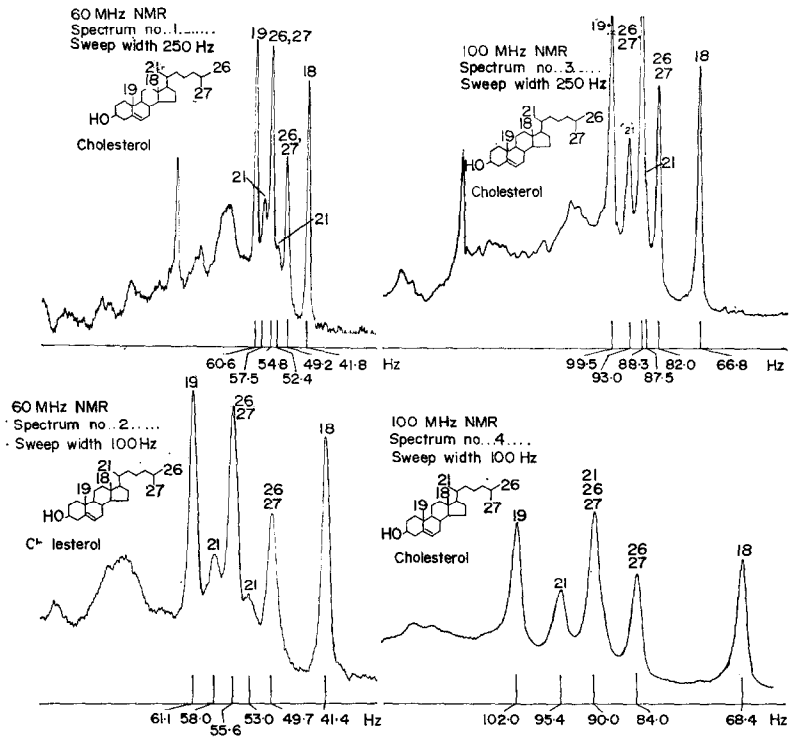

FIG. 1

Apparently, in campesterol, the α-oriented 28-methyl results in the 26 and 27-methyls being nonequivalent; thus, instead of just a doublet appearing for the 26 and 27-methyls as for cholesterol (spectrum 4), there are four peaks—doublets occurring at 82·0 and 88·8 Hz for the 26 or 27-methyl protons and at 77·0 and 83·8 Hz for the 27 or 26-methyl protons. In each case the coupling constant J is about 6·8 Hz and, generally, the coupling constants of methyl protons are in the range of 6–8 Hz. Interestingly, the 100 MHz spectrum 14 of 22,23-dihydrobrassicasterol shows mainly the expected doublet for the 26 and 27-methyl protons at 81·8 and 88·9 Hz along with miniature peaks at 76·7 and 83·5 Hz that occur as large peaks in the spectrum of campesterol. Perhaps in the 22,23-dihydrobrassicasterol

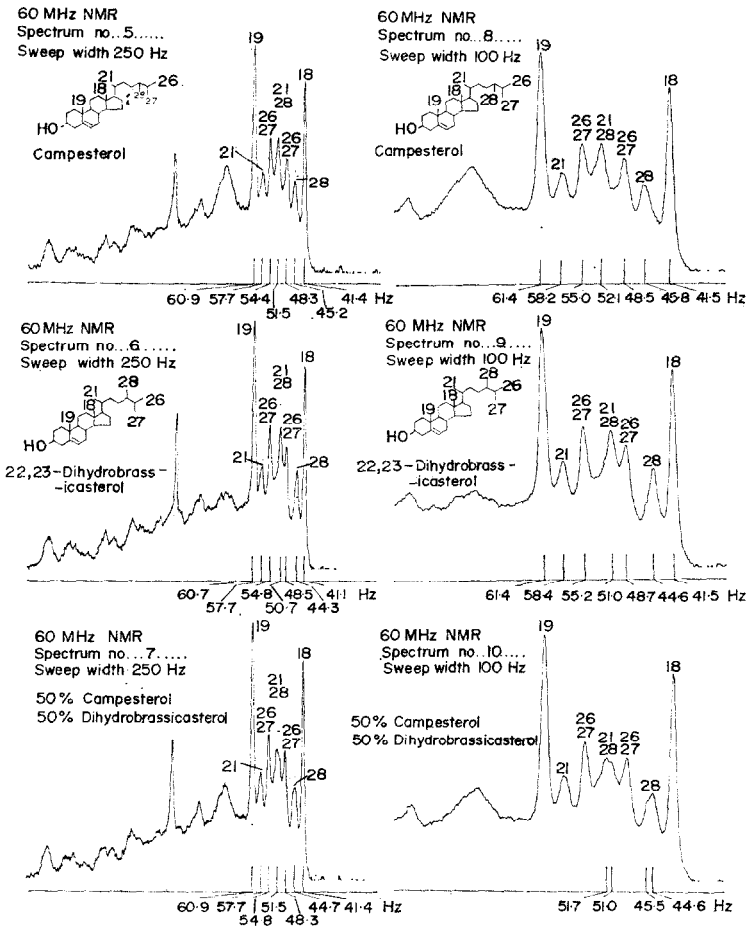

FIG. 2

the β-oriented 28-methyl results in only a small percentage of the 26 and 27-methyl groups being nonequivalent, hence only small peaks at 76·7 and 83·5 Hz. However, what we may be observing is simply a time scale 'average' spectral effect, that is the internal rotation of the terminal isopropyl group (C-26 and C-27 methyls) may, because of hindrance, be exceptionally slow in campesterol so as to bring about the nonequivalence of these methyls, hence the two sets of doublets for the 26 and 27 methyls. Additional NMR spectroscopic experiments are planned for 22,23-dihydrobrassicasterol and campesterol.

22,23-*Dihydrobrassicasterol from* Chlorella ellipsoidea

The 22,23-dihydrobrassicasterol used in this study was prepared from ergosterol via *i*-ergosterol.[3] The question has been asked whether it is possible that during the hydrogenation of the Δ^{22}-bond that one could have obtained a mixture of C-24 isomers. This we thought to be unlikely; though the NMR spectra of 22,23-dihydrobrassicasterol obtained from *Chlorella ellipsoidea* Gerneck[4] (spectra 16 and 26) do not show the minor peaks in

[3] M. J. THOMPSON, C. F. COHEN and S. M. LANCASTER, *Steroids* **5**, 745 (1965).
[4] G. W. PATTERSON and R. W. KRAUSS, *Plant and Cell Physiol.* **6**, 211 (1965).

the region of 76·7 and 83·5 Hz that occur in the spectra of synthetic 22,23-dihydrobrassicasterol (spectra 12 and 14). These peaks are major peaks in the spectrum of campesterol (spectra 11 and 13). Since the concentrations of the two sterols in determining the NMR spectra were different, (0·1 M for the synthetic and 0·025 M for the 22,23-dihydrobrassicasterol isolated from *C. ellipsoidea*), we thought that this difference in concentration could account for the differences in spectra. Yet, when the synthetic sterol was examined at a concentration of 0·025 M, its spectrum still showed the minor peaks at 76·7 and 83·5 Hz. This finding does suggest that during the hydrogenation of the Δ^{22}-bond in the preparation of 22,23-dihydrobrassicasterol (24β-methyl) a small amount of the 24α-isomer was formed. However, only the NMR spectra of naturally occurring 22,23-dihydrobrassicasterol obtained at a concentration of 0·1 M would ultimately answer this question.

These spectra (16 and 26) confirm the correctness of the original conclusion[4] that the No. 1 sterol from *C. ellipsoidea* is 22,23-dihydrobrassicasterol and not campesterol. It also demonstrates the feasibility of using NMR spectroscopy as a tool for ascertaining the configuration at C-24 with a small quantity of sterol (~4 mg/0·4 ml of solvent).

Conversion of 24-Methylenecholesterol to C-24 Isomeric Sterols

The NMR spectrum 17 obtained after the catalytic reduction of the Δ^{24}-bond of *i*-24-methylenecholesterol methyl ether in ethyl acetate over 10% palladium on charcoal and its subsequent conversion to the Δ^5-sterol showed that the expected mixture of C-24

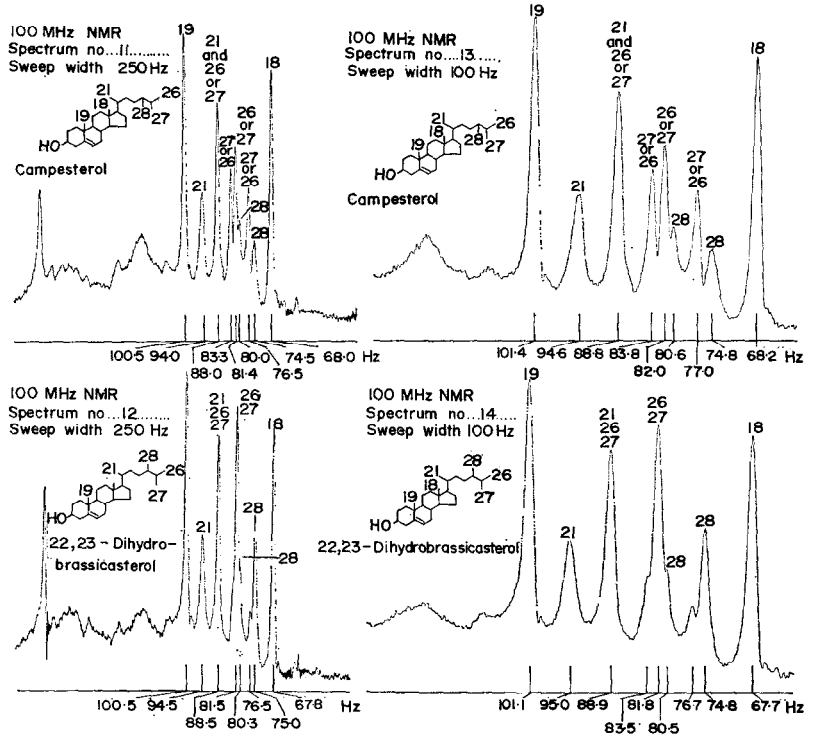

FIG. 3

isomeric sterols contained better than 50% of the 24β-methyl isomer (22,23-dihydrobrassicasterol). This conclusion is most obvious when the peaks at 76·8 and 83·4 Hz are compared with those of spectrum 18 (1:1 mixture of dihydrobrassicasterol and campesterol).

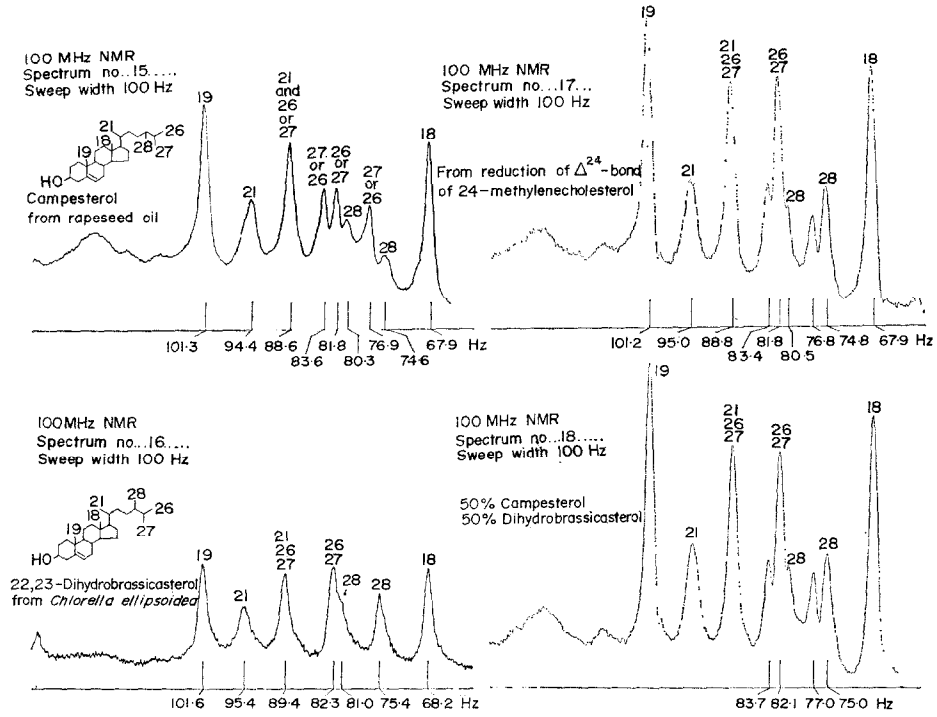

FIG. 4

Brassicasterol (24β-Methyl) and Campesterol (24α-Methyl) from Rapeseed Oil

The co-occurrence in rapeseed oil of sterols of opposite configuration at C-24, such as brassicasterol (24β-methylcholesta-5,22-dien-3β-ol) and campesterol,[5–7] infers a stereochemically heterogenous biosynthetic pathway (at C-24) for these two sterols. Though previous characterization of these sterols from rapeseed oil seemed conclusive, it was just possible that brassicasterol from rapeseed oil was not brassicasterol but was 22-dehydrocampesterol or that the campesterol from rapeseed oil was 22,23-dihydrobrassicasterol. Our findings with authentic samples of campesterol and 22,23-dihydrobrassicasterol indicated that this question could be answered by means of NMR spectroscopy. As in the conversion of stigmasterol to sitosterol,[8] brassicasterol was converted in a 50% overall yield to 22,23-dihydrobrassicasterol. Its NMR spectrum was identical to that of 22,23-dihydrobrassicasterol (spectrum 14) prepared from ergosterol, thus confirming that brassicasterol from the rapeseed oil was indeed brassicasterol and not 22-dehydrocampesterol. The very close agreement of NMR spectrum 15 to that of campesterol isolated from soybean oil spectrum 13 confirms that the campesterol from rapeseed oil was also indeed campesterol

[5] A. WINDAUS and A. WELSCH, *Chem. Ber.* **42,** 612 (1909).
[6] E. FERNHOLZ and H. E. STAVELY, *J. Am. Chem. Soc.* **62,** 428, 1875 (1940).
[7] E. FERNHOLZ and H. B. MACPHILLAMY, *J. Am. Chem. Soc.* **63,** 1155 (1941).
[8] J. A. STEELE and E. MOSETTIG, *J. Org. Chem.* **28,** 571 (1963).

and also demonstrates that campesterol isolated from two different sources exhibits identical NMR spectra.

Indeed, sterols of opposite configuration at C-24 do occur in the same seed. This confirmation by NMR spectroscopy permits us to conclude that the simultaneous biosynthesis of a $C_{28}\Delta^5$-sterol and a $C_{28}\Delta^{5,22}$-sterol in certain plants results in the production of sterols of opposite configuration at C-24 while that of a $C_{29}\Delta^5$-sterol and a $C_{29}\Delta^{5,22}$-sterol results in the production of sterols of identical configuration. The latter conclusion is reached by the observation that there are no known instances, as far as we know, of a $C_{29}\Delta^5$-sterol and $C_{29}\Delta^{5,22}$-sterol of opposite configuration at C-24 being isolated from the same source, i.e. sitosterol and stigmasterol occur together as does clionasterol and poriferasterol.

Stigmasterol (24α-Ethyl) and Poriferasterol (24β-Ethyl)

Stigmasterol and poriferasterol though exhibiting identical IR spectra are readily differentiated by their melting points. Their NMR spectra recorded at 60 MHz and at sweep widths of 250 and 100 Hz are indistinguishable from each other. However, their NMR spectra recorded at 100 MHz and at sweep width of 100 Hz are quite different in the 81–84 Hz region, with the peaks being better resolved in this region for stigmasterol (spectrum 19) than that of poriferasterol (spectrum 20). The remaining part of the spectra other than the 81–84 Hz region mentioned above are similar if not identical. The 21-methyl resonance peaks due to the Δ^{22}-bond are further down field than they are in the spectrum of cholesterol or the C_{28} sterols with the saturated side chains. The 18-methyl resonance is also further down field for this reason.

α-Spinasterol (24α-Ethyl) and Chondrillasterol (24β-Ethyl)

The IR and NMR spectra of α-spinasterol and chondrillasterol recorded at 60 MHz were also indistinguishable from each other. These two sterols are also the most difficult of the pairs we examined by NMR spectroscopy to distinguish by their physical properties. However, the NMR spectrum of chondrillasterol (spectrum 24) and α-spinasterol (spectrum 23) recorded at 100 MHz and sweep width 100 Hz are noticeably different in the 80–84 Hz region. The most noticeable differences are that the peak for the 19-methyl resonance (80·5 Hz) and part of the triplet (76·4 Hz) occurring farthest up field for the 29-methyl of chondrillasterol are separated by 4·1 Hz, while in the spectrum of α-spinasterol these corresponding peaks are only separated by 3 Hz. The 19-methyl resonance peak (80·5 Hz) and the middle peak of the triplet (82·5 Hz) of the 29-methyl resonance in the spectrum of chondrillasterol are separated by 2 Hz; these peaks in the spectrum of α-spinasterol are 3·3 Hz apart. The resonance peaks of the other methyls occur in similar positions in both spectra. The 21-methyl proton resonances as in stigmasterol are farther down field than those of the saturated side chain sterols due to the Δ^{22}-bond. The 19-methyl protons and the 18-methyl protons are shifted farther up field than are those of the Δ^5-sterols because of the stereochemistry at C-5(α) and the Δ^7-bond, respectively.

Sitosterol (24α-Ethyl) and 22,23-Dihydroporiferasterol (24β-Ethyl)

Of the four pairs of C-24 isomeric sterols examined by NMR spectroscopy, sitosterol (spectrum 21) and 22,23-dihydroporiferasterol (spectrum 22) are the only pair that cannot be differentiated from each other. There is a difference in the ratios of peak heights at 84·6 Hz and 86·6 Hz region; however, such differences can occur in different scans of the same material. Such changes can also occur because of different instruments, operators, or

experimental conditions. This is illustrated by comparing spectrum 15 (campesterol from rapeseed oil) with spectrum 13; these spectra are similar except for the differences in ratios of peak heights at about the 81·8 and 83·6 Hz region. Although the NMR spectra of 22,23-dihydroporiferasterol (clionasterol) and sitosterol are the same, these sterols can be differentiated as their acetate by melting point-determinations—clionasterol acetate melts at 143° while sitosterol acetate melts at 122°.

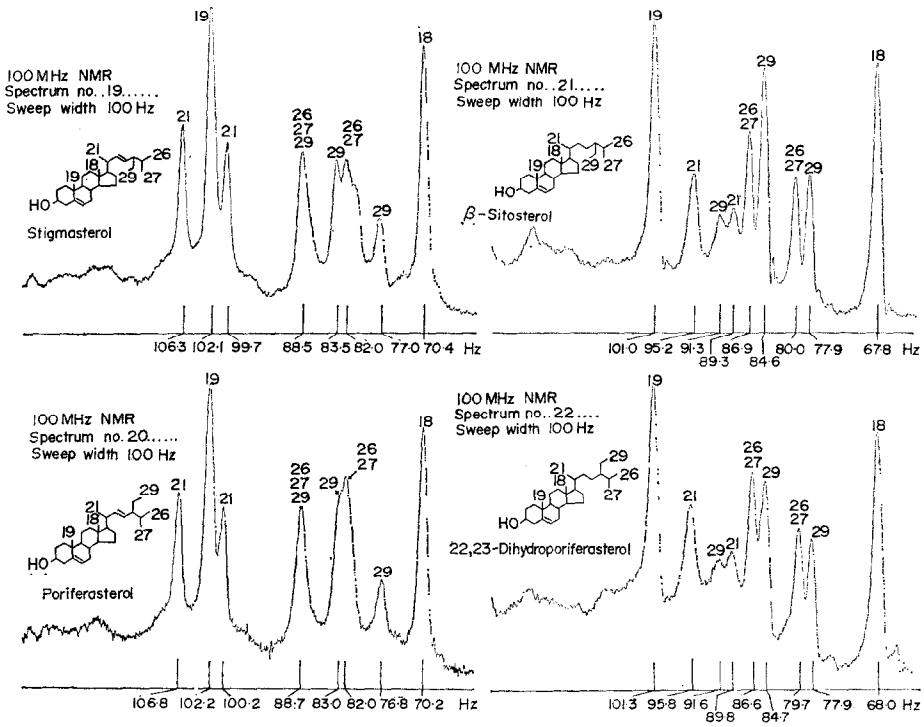

FIG. 5

In the NMR spectra of sitosterol and 22,23-dihydroporiferasterol, the triplet for the 29-methyl occurs at 77·9, 84·6 and 91·3 Hz. However, the two upfield peaks at 77·9 and 84·6 Hz are considerably larger than would be expected for the 29-methyl protons alone. Since these two peaks occur in the same region as the upfield doublet found in the spectrum of campesterol (spectrum 13) attributed to the 27 or 26-methyl protons, it seems most likely that a similar situation prevails in spectra 21 and 22. The contribution of the 27- or 26-methyl protons to the upfield 29-methyl peaks would explain the abnormal heights and areas of these peaks. This explanation should be confirmed by examining these two sterols with a 220 MHz instrument.

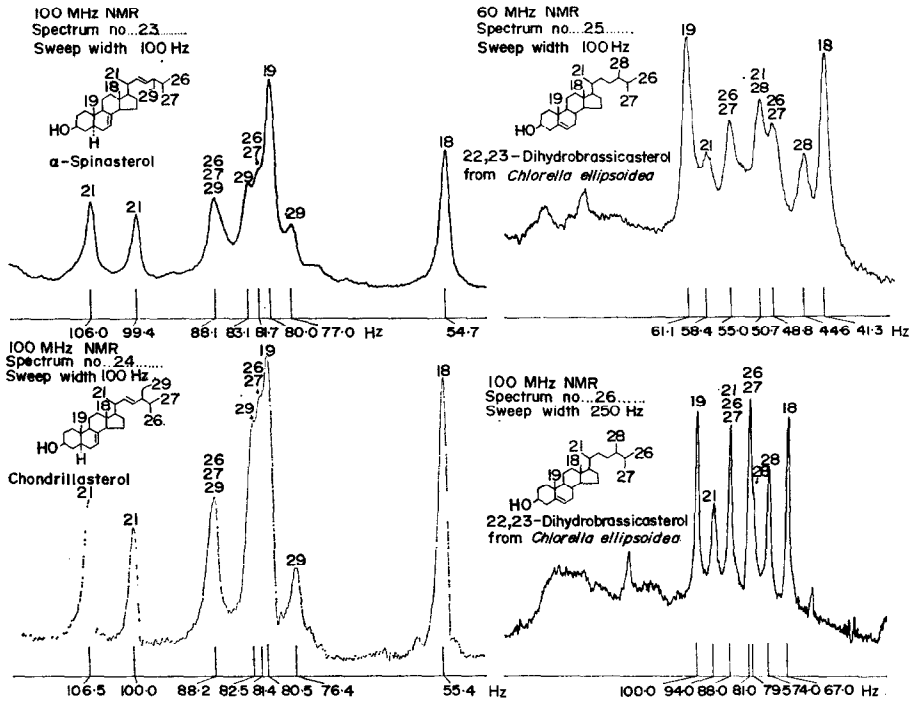

FIG. 6

EXPERIMENTAL

M.ps were taken on a Kofler block and are corrected. Rotations were determined on approximately 1% solutions in $CHCl_3$ at 23° and IR were obtained in CS_2. The NMR spectra were recorded at 60 and 100 MHz with Varian A-60A and HA-100 NMR spectrometers, respectively, using $CDCl_3$ as solvent and TMS as internal standard. The positions of the peaks in the spectra are given in Hz and can be converted to ppm by dividing the values by 60 or 100 for the 60 or 100 MHz, respectively. The concentration of the solutions were approx. 0·1 M except for the sample of 22,23-dihydrobrassicasterol obtained from *Chlorella ellipsoidea* Gerneck, i.e. spectra 16 and 26, the concentration was 0·025 M. The temperature of the solutions during the recording of the spectra were 44 and 33° with the A-60A and HA-100 NMR spectrometers, respectively. Analyses by GLC to determine the purity of the sterols were obtained on Barber–Colman Model 10 chromatograph using columns of 0·75% SE-30 and 1% QF-1. The purity of the sterols were also evaluated by TLC on Silica Gel plates and $AgNO_3$-impregnated Silica Gel H plates. The purity of the sterols used in this study were greater than 99% unless otherwise stated. The NMR spectra of cholesterol recorded at 60 and 100 MHz and sweep widths of 100 and 250 Hz have been included for comparative interpretive purposes.

Sterols used in the study. Cholesterol (m.p. 149–150°, $[\alpha]_D$ −40°) from Fisher Scientific Co. after two recrystallizations from MeOH was used without any further purification. Sitosterol (m.p. 139–140°, $[\alpha]_D$ −34°) was prepared from stigmasterol according to the procedure of Steele and Mosettig.[8] Brassicasterol (m.p. 149–151°, $[\alpha]_D$ −66°) and 22-23-dihydrobrassicasterol (m.p. 158–159°, $[\alpha]_D$ −44°) were prepared from ergosterol as previously reported by Thompson *et al.*[3] Campesterol (> 98% purity, m.p. 160–161°, $[\alpha]_D$ −33°) was obtained by fractional crystallization from acetone of soybean sterols from which stigmasterol had been removed. Stigmasterol (m.p. 169–170°, $[\alpha]_D$ −50°) was a gift from the Upjohn Co., Kalamazoo, Michigan. Poriferasterol (m.p. 157–158°, $[\alpha]_D$ −55°) was isolated from *Chlorella ellipsoidea*[4] and chondrillasterol (m.p. 169–170°, $[\alpha]_D$ ±0°) from *Chlorella emersonii* var. *emersonii* Shihira and Kraus.[4,9] 22,23-Dihydroporiferasterol (m.p. 142–143°) was prepared from poriferasterol as in the preparation of β-sitosterol from stigmasterol. α-Spinasterol (m.p. 168–169°, $[\alpha]_D$ −4°) was obtained from alfalfa and the 24-methylenecholesterol (m.p. 144–146°, $[\alpha]_D$ −36°) from pollen collected from the saguaro cactus (*Carnegiea gigantea* (Engelm.) (Britt & Rose).

Conversion of Brassicasterol from Rapeseed Oil to 22,23-Dihydrobrassicasterol. The rapeseed oil obtained from Stevenson Brothers and Co., Philadelphia, Pa., was saponified in the presence of 10% KOH in 75%

[9] G. W. PATTERSON, *Plant Physiol.* **42**, 1457 (1967).

ethanol at reflux temp. in N_2 for 5 hr. Most of the alcohol was removed under vacuum and the nonsaponifiable material was extracted into Et_2O. The nonsaponifiable material was separated into a sterol fraction by the fractionation of 1·0 g of material/30 g of hexane-washed neutral alumina (Woelm, activity grade II) and eluting with 200 ml each of hexane, hexane–benzene (1:1), benzene, and Et_2O. The sterols which were eluted in the Et_2O fraction were acetylated and the brassicasterol acetate was isolated by repeated column chromatography on 20% $AgNO_3$ impregnated silica gel. The fractions were monitored by GLC and those exhibiting a single peak with a relative time identical to that of brassicasterol acetate were combined and recrystallized twice from Et_2O–MeOH to give brassicasterol acetate, m.p. 154–155°. Its saponification with 2% K_2CO_3 in 90% MeOH gave, after two recrystallizations from dilute acetone, brassicasterol, m.p. 147·5–149°, $[\alpha]_D$ −67°. The brassicasterol, as in the conversion of stigmasterol to sitosterol,[8] was converted in a 50% overall yield to 22,23-dihydrobrassicasterol, m.p. 159–160°, $[\alpha]_D$ −45°. Its NMR spectrum was identical to that of 22,23-dihydrobrassicasterol (spectrum 14) prepared from ergosterol, thus confirming that brassicasterol from the rapeseed oil was indeed brassicasterol and not 22-dihydrocampesterol.

Campesterol from rapeseed oil. Fractional crystallization from acetone of the 7:13 mixture of C_{28} and C_{29} Δ^5-sterols from the rapeseed oil yielded campesterol (C_{28} sterol) of about 96% purity, m.p. 159–160°, $[\alpha]_D$ −33°. Its NMR spectrum (spectrum 15) was also identical with that of campesterol (spectrum 13) isolated from soybean oil, thus confirming that Δ^5-sterols isomeric at C-24 (brassicasterol and campesterol) do occur in the same seed.

Acknowledgements—We thank Dr. L. M. Twannoh of the National Cancer Institute, Bethesda, Md., for several of the NMR spectra and Dr. L. N. Standifer of the USDA Honey Bee Research Laboratory, Tucson, Arizona, for the pollen collected from the saguaro cactus.

Key Word Index—C-24 isomeric sterols; NMR spectroscopy; distinction between isomers.