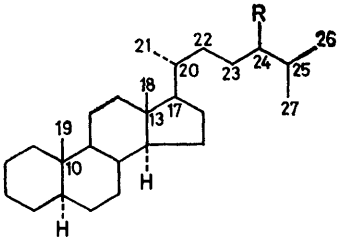
Proton Magnetic Resonance Studies of Sterols. Assignment of Methyl Signals of C_{27}, C_{28}, C_{29} Analogues

By **Thomas A. Wittstruck,* Jacek K. Sliwowski, and Eliahu Caspi,** Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, U.S.A.

The ^{1}H n.m.r. chemical shifts, obtained at 100 MHz, of a number of cholestane, ergostane, and stigmastane analogues have been assigned. Deviations from additivity of substituent effects upon the angular methyl chemical shifts were noted for certain 14,15 disubstituted compounds. Resolution of signals from the C-25 methyl groups of stigmastanes, but not of the ergostanes, was observed.

In the course of biosynthetic studies, it became necessary to assign the ^{1}H n.m.r. signals of a number of compounds of the cholestane (1a), ergostane (1b), and stigmastane (1c) series.[1] The ^{1}H n.m.r. spectra of cholestane and its



(1) a; R = H
b; R = $^{28}CH_3$
c; R = $^{28}CH_2{}^{29}CH_3$

derivatives have been reported on numerous occasions. Similarly, the numerous data on the effects of certain ring substituents on the chemical shifts of 10- and 13-methyl groups of sterols in general greatly facilitate the assignment of peaks for these compounds.[2] However, when additional alkyl groups are present and/or the ring system contains functions whose substituent effects on the 10- and 13-methyl groups are not known, the assignment of the methyl peaks for such compounds is complicated. We now report the assignment of the methyl signals (at 100 MHz) of the C_{27}, C_{28}, and C_{29} steroids listed in Table 1.

The compounds used in this study were synthesized in our laboratory and were fully characterized.[1] The ^{1}H n.m.r. spectra were obtained from solutions in $CDCl_3$. The spectra of compounds (2), (7), (12), and (18), were also recorded for solutions in C_6H_6. In each case, tetramethylsilane was used as internal reference.

Table 1

Compounds investigated

(1)	5α-Cholestane
(2)	5α-Cholestan-3β-yl acetate
(3)	5α-Cholest-9(11)-en-3β-yl acetate
(4)	5α-Cholest-14-en-3β-yl acetate
(5)	14α,15α-Epoxy-5α-cholestan-3β-yl acetate
(6)	5α-Cholestane-3β,14α,15β-triol 3-acetate
(7)	3β-Acetoxy-14α-hydroxy-5α-cholestan-15-one
(8)	5α-Ergost-8(14)-en-3β-yl acetate
(9)	5α-Ergost-14-en-3β-yl acetate
(10)	14α,15α-Epoxy-5α-ergostan-3β-yl acetate
(11)	5α-Ergostane-3β,14α,15β-triol 3-acetate
(12)	3β-Acetoxy-14α-hydroxy-5α-ergostan-15-one
(13)	5α-Stigmastan-3β-yl acetate
(14)	5α-Stigmast-9(11)-en-3β-yl acetate
(15)	5α-Stigmast-14-en-3β-yl acetate
(16)	14α,15α-Epoxy-5α-stigmastan-3β-yl acetate
(17)	5α-Stigmastane-3β,14α,15β-triol 3-acetate
(18)	3β-Acetoxy-14α-hydroxy-5α-stigmastan-15-one
(19)	5α-Stigmast-1-en-3-one
(20)	3β-Hydroxystigmast-5-en-7-one
(21)	Stigmast-5-en-3β-ol-7α-hydroperoxide

The spectra were recorded with a Varian HA-100-15 spectrometer, at room temperature. Each spectrum was first recorded at 1 000 Hz sweep width; and then the high field portion was recorded at 250 Hz sweep width. The frequencies of the methyl signals were measured to the nearest 0.1 Hz from the 250 Hz spectra. All other signals were measured, by using the 1 000 Hz spectra, to the nearest 0.4 Hz. First-order analysis of

[1] (*a*) E. Caspi, J. P. Moreau, and P. J. Ramm, *J. Amer. Chem. Soc.*, 1974, **96**, 8107 (C_{27} series); (*b*) J. P. Moreau, P. J. Ramm, and E. Caspi, *European J. Biochem.*, 1975, **56**, 393 (C_{27} series); (*c*) E. Caspi, J. Sliwowski, and C. S. Robichand, *J. Amer. Chem. Soc.*, 1975, **97**, 3820 (C_{29} series); (*d*) J. K. Sliwowski and E. Caspi, *J.C.S. Chem. Comm.*, 1976, 196 (C_{29} series); (*e*) J. K. Sliwowski, V. R. Reddy and E. Caspi, *J. Neurochem.*, in the press (C_{27} series); (*f*) J. K. Sliwowski and E. Caspi, *J. Amer. Chem. Soc.*, in the press (C_{29} series); (*g*) J. K. Sliwowski and E. Caspi, submitted for publication (C_{28} series).

[2] (*a*) E. Caspi and T. A. Wittstruck, 'Steroid Hormone Analysis,' vol. 1, ed. H. Cartensen, Dekker, New York, 1967, ch. 3; (*b*) N. S. Bhacca and D. H. Williams, 'Applications of NMR Spectroscopy in Organic Chemistry,' Holden–Day, San Francisco, 1964; (*c*) W. Arnold, W. Meister, and G. Englert, *Helv. Chim. Acta*, 1974, **57**, 1559.

the multiplets observed for the side-chain methyl signals was performed.

Side-chain Methyl Signals.—The chemical shifts of the side-chain methyl protons of the cholestanes (Table 2, entries 1—7) were assigned without difficulty. The secondary 20- and 25-methyl groups produce a doublet each. However, the signals from the 25-methyl groups of cholestane coincide at 100 MHz, and the resulting doublet corresponds in intensity to six protons.[3] In general, the 20-methyl signal of cholestane and its derivatives is sufficiently displaced from those for the 25-methyl signals so that unambiguous assignments can be made.

The effects of various ring substituents upon the chemical shifts of the side-chain methyl protons of the cholestanes are listed in Table 3. In most instances the effects are small, but they are not negligible. As expected, the ring-substituent effects are greater at the 20-methyl than at the 25-methyl groups.

In the 100 MHz ^{1}H n.m.r. spectra of the ergostane analogues (Table 2, entries 8—12), the 25-methyl signals also overlap, and give a six-proton doublet. The chemical shifts of the 20- and 24-methyl groups were assigned on the basis of comparisons with spectra of appropriate ergostane and stigmastane derivatives (see later).

In the spectra of stigmastane derivatives (Table 2; entries 13—21), the 25-methyl signals *are* resolved into two doublets, separated by *ca.* 0.02 p.p.m. The detected three-proton triplet most certainly arises from the protons at C-29. By elimination the third methyl doublet is assigned to the 20-methyl group.

As a first approximation, it may be assumed that the effect of the 24-ethyl group of the stigmastanes and the 24-methyl group of the ergostanes upon their 20-methyl signals will be nearly the same. Therefore, the chemical shifts of the 20-methyl groups in the ergostane and stigmastane series should be similar. By comparing the spectra of analogous stigmastane and ergostane derivatives, the assignments of the 20-methyl signals in the spectra of the ergostane derivates were made. The unassigned doublet in the spectra of the ergostane derivatives (see above) was ascribed to the 24-methyl group.

TABLE 2

^{1}H N.m.r. chemical shifts and coupling constants [a]

Cholestanes

Compd.	13-CH_3	10-CH_3	20-CH_3	25-$(CH_3)_2$	3β-OAc	3α-H [b]	Others
(1)	0.645	0.778	0.898 (6.0)	0.861 (6.3)			
(2)	0.648	0.820	0.900 (6.5)	0.860 (6.5)	1.985	4.68	
(3)	0.570	0.946	0.892 (6.0)	0.852 (6.0)	1.980	4.68	5.24 (11-H)
(4)	0.898	0.838	0.910 (6.0)	0.868 (6.0)	2.010	4.69	5.12 (15-H)
(5)	0.858	0.858	0.864 (6.3)	0.864 (6.3)	2.000	4.68	3.30 (15β-H)
(6)	0.790	0.998	0.925 (6.0)	0.866 (6.5)	1.990	4.68	4.02 (15α-H)
(7)	0.798	1.035	0.948 (6.0)	0.869 (6.3)	2.000	4.68	

Ergostanes

Cmpd.	13-CH_3	10-CH_3	20-CH_3	25-$(CH_3)_2$	24-CH_3	3β-OAc	3α-H [b]	Others
(8)	0.845	0.710	0.878 (6.5)	0.789 (6.8)	0.940 (6.0)	2.000	4.68	
(9)	0.885	0.825	0.855 (6.0)	0.778 (6.5)	0.905 (6.0)	2.000	4.68	5.13 (15-H)
(10)	0.848	0.848	0.790 (6.0)	0.776 (6.8)	0.845 (6.0)	1.990	4.67	3.30 (15β-H)
(11)	0.778	0.990	0.850 (6.5)	0.780 (6.5)	0.918 (6.0)	2.000	4.67	4.02 (15α-H)
(12)	0.798	1.030	0.860 (6.5)	0.790 (6.5)	0.948 (6.0)	1.980	4.68	

Stigmastanes

Cmpd.	13-CH_3	10-CH_3	20-CH_3	25-$(CH_3)_2$	28-CH_3	3β-OAc	3α-H [b]	Others
(13)	0.660	0.824	0.910 (6.0)	0.810 (6.0), 0.836 (6.8)	0.920 (6.0)	2.010	4.68	
(14)	0.590	0.960	0.908 (6.5)	0.815 (6.5), 0.835 (6.5)	0.913 (6.0)	2.010	4.68	5.24 (11-H)
(15)	0.890	0.838	0.860 (6.5)	0.804 (6.5), 0.826 (6.8)	*c*			
(16)	0.855	0.855	*c*	0.813 (6.5), 0.833 (6.5)	*c*	1.990	4.66	3.30 (15β-H)
(17)	0.783	0.995	0.925 (6.0)	0.818 (6.5), 0.828 (6.5)	0.905 (6.0)	2.000	4.68	4.04 (15α-H)
(18)	0.788	1.020	0.938 (6.5)	0.810 (6.5), 0.825 (6.5)	0.920 (6.0)	2.000	4.68	
(19)	0.705	1.010	0.928 (6.0)	0.823 (6.5), 0.844 (6.8)	0.920 (6.0)			5.83 (10.0, 2-H); 7.12 (10.0, 1-H)
(20)	0.685	1.198	0.923 (6.5)	0.814 (6.8), 0.835 (6.5)	0.915 (6.0)		3.62	4.67 (6-H)
(21)	0.695	1.203	0.966 (6.8)	0.821 (6.8), 0.843 (6.5)	0.920 (6.0)		3.6	4.69 (6-H); 3.3 (7β-H)

[a] Chemical shifts in p.p.m. (±0.001); coupling constants (in parentheses) in Hz; solvent $CDCl_3$. [b] Approximate centre of broad multiplet. [c] Peaks not observed, overlapped by other signals.

TABLE 3

Effects of substituents upon methyl signals [a]

Substituent	20-CH_3	25-$(CH_3)_2$	10-CH_3	13-CH_3
9(11)-Ene	−0.008	−0.008	0.126	−0.078
14-Ene	0.010	0.008	0.018	0.250
14α,15α-Epoxy	−0.036	0.004	0.038	0.210
14α,15α-Dihydroxy	0.023	0.006	0.178	0.142
14α-Hydroxy-15-oxo	0.048	0.009	0.215	0.150
3β-Acetoxy	0.002	−0.001	0.042	0.003
24-Methyl	−0.081	−0.083	−0.009	−0.008
24-Ethyl	−0.030	−0.062, −0.044	−0.008	−0.009

[a] In p.p.m.; a negative sign implies an upfield displacement.

[3] G. Slomp and F. A. Mackellar, *J. Amer. Chem. Soc.*, 1962, **84**, 204.

10- *and* 13-*Methyl Signals*.—Except for the 14α,15β-dihydroxy- and 14α-hydroxy-15-oxo-analogues, assignment of the 10- and 13-methyl chemical shifts was straightforward. Usually the observed and calculated chemical shifts agreed within *ca.* ±0.01 p.p.m.

Initially, estimates of the 10- and 13-methyl chemical shifts of 3β-acetoxy-14α-hydroxy-5α-cholestan-15-one (7) were made by adding the individual substituent effects [2] of 14α-hydroxy and 15-oxo to the 10- and 13-methyl chemical shifts of 5α-cholestan-3β-yl acetate (2). However, these estimated values (0.840 p.p.m. for the 13- and 0.828 p.p.m. for the 10-Me) do not have corresponding signals in the spectrum of (7). This lack of agreement was not unexpected. Electrical characteristics of individual functions in a molecule may be modified if these functions interact,[2b] and thus deviations from additivity of their influence may occur.

To differentiate the 10- and 13-methyl signals of compound (7) and its analogues, spectra of solutions in benzene were recorded (Table 4). For molecules containing a carbonyl group, because of complexation of the carbonyl with benzene, the signals of protons in the plane of the complexing benzene will be shifted downfield.[2b] Conversely the signals of protons located in the shielding cone of the complexing benzene will be shifted upfield.

TABLE 4

^{1}H N.m.r. chemical shifts and coupling constants for solutions in benzene [a]

Cmpd.	13-CH_3	10-CH_3	20-CH_3	25-$(CH_3)_2$	24-CH_3	3β-OAc
(2)	0.643	0.693	1.000 (6.5)	0.923 (6.0)		1.750
(7)	0.558	0.978	0.905 (6.5)	0.945 (6.5)		1.760
(12)	0.560	0.978	0.910 (6.5)	0.876 (6.8)	0.944 (6.8)	1.760
(18)	0.553	0.993	*b*	*b*	*b*	1.750

[a] Chemical shifts in p.p.m. (±0.001); coupling constants (in parentheses) in Hz. [b] Peaks not recorded.

The signals for the 20-, 25-, and acetate methyl groups of (7) in benzene were assigned without difficulty. The acetate signal is shifted 0.240 p.p.m. upfield from its position in the $CDCl_3$ spectrum. This is in good agreement with the reported upfield displacement of 0.23 p.p.m. for the 3β-OAc signal of 5,6-dihydroergosterol 3β-acetate.[2b] The benzene-induced shifts of the 20- and 25-methyl signals are −0.043 and 0.076 p.p.m., respectively. It is not obvious which of the two signals at 0.558 and 0.978 p.p.m. in the spectrum of (7) in benzene should be assigned to the 10- and which to the 13-methyl. The following arguments were used to differentiate these two signals.

It is reasonable to assume that solute–solvent complexation occurs at both the 3β-acetate carbonyl and the C-15 carbonyl group of (7). In addition, the observed benzene-induced shift for each methyl signal may be assumed to be the sum of the contributions from complexation at each site. Thus, the displacement arising from complexation at the C-15 carbonyl of (7) may be obtained by subtracting the contribution of the benzene complexation with the 3β-acetate from the total benzene-induced shift. In order to estimate the contribution from complexation at the 3β-acetate, we measured the effect of solvent change on the signals of 5α-cholestan-3β-yl acetate (2). The benzene-induced shifts (p.p.m.) were 13-CH_3 −0.005; 10-CH_3 −0.127; 20-CH_3 0.100; 25-(CH_3) 0.063; and 3β-OAc −0.235. Molecular models indicate that complexation at the C-15 carbonyl of (7) would be expected to shift the 13- and 20-methyl signals upfield, and the 10- and 25-methyl signals downfield. As indicated above, complexation of benzene at *both* the 3β-acetate and the C-15 carbonyl shifted the 20- and 25-methyl signals of (7) −0.043 and 0.076 p.p.m., respectively. By subtracting from these values the contributions of the 3β-acetate, we obtain −0.143 p.p.m. (an upfield displacement) for the 20-methyl signal and 0.013 p.p.m. (a downfield displacement) for the 25-methyl signals. The direction of both benzene-induced shifts agrees with that predicted from the molecular models. The same procedure was used to determine the 10- and 13-methyl chemical shifts of (7). However, since it was not known *a priori* which signal of (7) corresponds to the 13- and which to the 10-methyl, two options were available for the calculations:

(a) 0.978 − (−0.005) = 0.983 p.p.m. (13) and
0.558 − (−0.127) = 0.685 p.p.m. (10)
(b) 0.558 − (−0.005) = 0.563 p.p.m. (13) and
0.978 − (−0.127) = 1.105 p.p.m. (10)

These ' corrected ' values were then compared with the values in $CDCl_3$. Once again it was not known which of the two signals of (7) in $CDCl_3$ corresponds to which methyl group. Therefore the peaks were compared in four different ways as shown schematically in Figure 1. Two of the possibilities [represented by (b) and (c)] are ruled out because they require the wrong direction of benzene-induced shift for one or both methyl signals. The remaining two possibilities, (a) and (d), both indicate that in the spectrum of (7) in benzene the signal at 0.558 p.p.m. must be assigned to the 13- and that at 0.978 p.p.m. to the 10-methyl group. The interpretation we prefer assigns the 0.798 and 1.035 p.p.m. signals in the $CDCl_3$ spectrum of (7) to the 13- and the 10-methyl groups, respectively. The benzene-induced shifts arising from the C-15 carbonyl are thus −0.235 p.p.m. for the 13-methyl and +0.070 p.p.m. for the 10-methyl group. The alternative assignment (d) would necessitate larger benzene-induced shifts (−0.472 and +0.307 p.p.m. for the 13- and 10-methyls, respectively). A benzene-induced shift of 0.307 p.p.m. for the 10-methyl group for complexation of benzene with the C-15 carbonyl seems unreasonable, especially

in view of the benzene-induced shift of only 0.013 p.p.m. noted above for the 25-methyl groups.

On the basis of the described assignments for the 10- and 13-methyl groups of (7), the assignments for

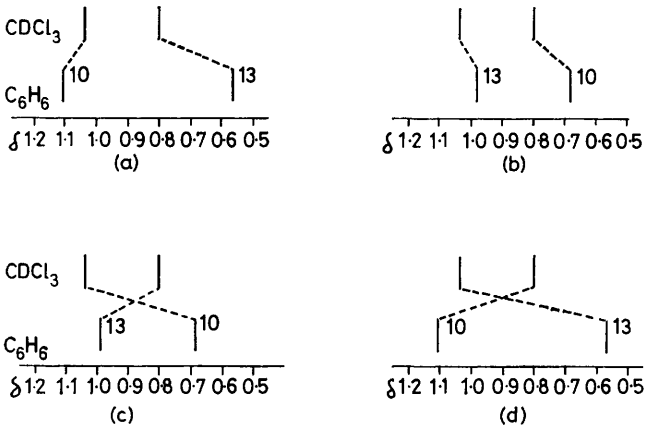


FIGURE 1 The four alternative ways of matching the angular methyl signals of 3β-acetoxy-14α-hydroxy-5α-cholestan-15-one (7) recorded in $CDCl_3$ and C_6H_6; the signals in C_6H_6 were corrected to remove the effects of complexation of benzene with the 3β-acetate group (see text)

the corresponding methyl groups of compounds (12) and (18) were made.

The observation that the 25-methyl signals of the stigmastane derivatives are resolved could be rationalized as follows. It may be assumed *a priori* that in general ' staggered ' configurations about a carbon–carbon bond

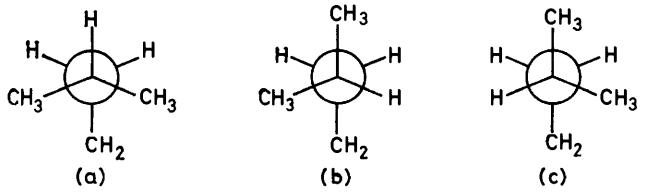


FIGURE 2 The three preferred rotamers about the 24,25-bond of cholestane; rotamers (b) and (c) are energetically equivalent

are more stable than the ' eclipsed ' configurations. It follows that the preferred configurational isomers corresponding to rotation about the 24,25-bond can be represented by the Newman projections shown in Figures 2 and 3. In the cholestane derivatives, two of these three configurations are equivalent [Figures 2(b) and (c)]. In the ergostanes and stigmastanes no two of the three configurations are equivalent (Figure 3). The 25-methyl chemical shifts for the three preferred rotamers can be symbolized by $\delta(26)_a$, $\delta(26)_b$, $\delta(26)_c$; and $\delta(27)_a$, $\delta(27)_b$, $\delta(27)_c$. Figure 2 shows that for cholestane the four chemical shifts $\delta(26)_a$, $\delta(26)_c$, $\delta(27)_a$, and $\delta(27)_b$ are equivalent. Similarly the two chemical shifts $\delta(26)_b$ and $\delta(27)_c$ are equivalent. Furthermore, if the fractional populations of the three rotamers are n_a, n_b, and n_c, from Figure 2 it is clear that n_b is equal to n_c, but not to n_a.

At the extreme of slow rotation about the 24,25-bond of cholestane, two methyl signals would be anticipated.[4] The chemical shifts would be $\delta(26)_a$ and $\delta(26)_b$, with relative intensities $(n_a + n_b)$ and n_b, respectively. For rapid rotation about the 24,25-bond, a single signal corresponding in intensity to both the 25-methyl groups is expected.[4] The observation of only a single signal (*i.e.* one doublet) for the cholestane derivatives at room temperature indicates either that rapid rotation about the 24,25-bond occurs, or that, if rotation is restricted, the chemical shift difference between the signals arising from the methyls in the different configurations is too small to be detected at 100 MHz.

Figure 3 shows that in ergostanes and stigmastanes $\delta(26)_a$ is equivalent to $\delta(27)_b$, $\delta(26)_b$ is equivalent to $\delta(27)_c$, and $\delta(26)_c$ is equivalent to $\delta(27)_a$. *A priori* it follows that n_a, n_b, and n_c (Figure 3) may be unequal.

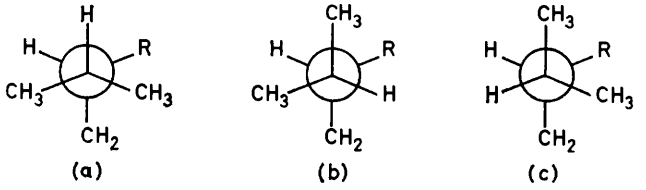


FIGURE 3 The three preferred rotamers about the 24,25-bond of ergostane (R = Me) and stigmastane (R = Et); no two of the three are energetically equivalent

Thus, for slow rotation about the 24,25-bond, the ergostanes and stigmastanes will produce three separate methyl signals at $\delta(26)_a$, $\delta(26)_b$, and $\delta(26)_c$, whose relative intensities should be proportional to n_a, n_b, and n_c, respectively.[4] The separation between the signals would depend upon how closely equivalent are the methyl groups in the three rotamers. For rapid rotation about the 24,25-bond, two signals of equal intensity will be expected. The anticipated chemical shifts of the signals are $1/3\ [n_a\ \delta(26)_a + n_b\ \delta(26)_b + n_c\ \delta(26)_c]$ p.p.m. and $1/3[n_b\ \delta(26)_a + n_c\ \delta(26)_b + n_a\ \delta(26)_c]$ p.p.m.[4] Since two doublets of equal intensity are seen for the stigmastane derivatives, this indicates that rapid rotation occurs about the 24,25-bond of these compounds. If this is so, it seems reasonable to assume that such is also the case for the cholestanes and ergostanes. As indicated above, the spectra of the cholestanes are consistent with the premise of rapid rotation. However, for the ergostane derivatives only a single doublet was seen for the 25-methyl groups together. It would appear therefore that a fortuitous equivalence occurs between the time-averaged chemical shifts of the two 25-methyl groups of the ergostanes.

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[4] J. A. Pople, W. G. Schneider, and H. J. Bernstein, ' High-resolution Nuclear Magnetic Resonance,' McGraw-Hill, New York, 1959, p. 377.