Proton nuclear magnetic resonance identification and discrimination of side chain isomers of phytosterols using a lanthanide shift reagent

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Abstract Proton nuclear magnetic resonance (^{1}H-NMR) spectra at 90 MHz were measured for a number of side chain isomers of phytosterols (sterols with a C_8H_{17} side chain, and Δ^{24}-, 24-methylene, Δ^{22}-, 24-ethylidene, 24-methyl, 24-ethyl, 24-methyl-Δ^{22}-, 24-ethyl-Δ^{22}-, and 24-ethyl-$\Delta^{22,25(27)}$-sterols) with or without a lanthanide shift reagent, tris[1,1,1,2,2,3,3 - heptafluoro - 7,7 - dimethyloctane - 4,6 - dionato]ytterbium, Yb(fod)$_3$, and the effect of Yb(fod)$_3$ on the side chain methyl protons is discussed. The change of the chemical shifts induced by Yb(fod)$_3$ for the side chain methyls was expressed in terms of the induced shift ratios (ISR values), i.e., the ratios of the induced chemical shifts of the respective side chain methyls to that of the fastest moving side chain methyl. The ISR values were sensitive to minor structural and stereochemical differences, but almost independent of ring structures and of substrate concentrations, thus providing confirmatory evidence for the side chain structures. Also, the Yb(fod)$_3$-induced spectral patterns observed in the high-field methyl region bore the fingerprints of the side chain structures. Several isomeric pairs of sterols, which differ only in the geometry of double bonds or the absolute configuration at C-24 in the side chain, i.e., *cis*- and *trans*-isomers of Δ^{22}- and 24-ethylidene sterols, 24*R*/α- and 24*S*/β-methyl sterols, 24*R*/α- and 24*S*/β-ethyl sterols, and 24*S*/α- and 24*R*/β-ethyl-Δ^{22}-sterols, could be differentiated from each other under the influence of Yb(fod)$_3$, even though they were measured at 90 MHz.—**Iida, T., T. Tamura, and T. Matsumoto.** Proton nuclear magnetic resonance identification and discrimination of side chain isomers of phytosterols using a lanthanide shift reagent. *J. Lipid Res.* 1980. **21:** 326–338.

Supplementary key words Yb(fod)$_3$ · induced chemical shifts · induced shift ratios · *cis*- and *trans*-isomers of sterols · C-24 epimers of C-24 alkylated sterols · absolute configuration at C-24

There are a variety of phytosterols in natural sources (1, 2), which differ from one another merely by the presence or absence of unsaturated bonds and alkyl substituents and their locations in the side chain at C-17 position. Perhaps proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is one of the most powerful means for their structural identification. In fact, a number of natural and synthetic sterols related to cholestane, ergostane, and stigmastane series have hitherto been analyzed using a 40, 60, 90, or 100 MHz ^{1}H-NMR spectrometer (3–8), and the chemical shift data obtained therefrom are of particular importance in elucidating the structure of an unknown sterol. However, investigators, in using these instruments, have often encountered the problem of distinguishing between isomeric phytosterols differing only in the stereochemistry of an unsaturated bond or an alkyl

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; LSR, lanthanide shift reagent; Yb(fod)$_3$, tris[1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato]ytterbium; LIS, lanthanide-induced shift; ISR values, induced shift ratios. Trivial names and their corresponding systematic names are as follows: cholestanol, 5α-cholestan-3β-ol; cholesterol, cholest-5-en-3β-ol; lathosterol (or Δ^7-cholestenol), 5α-cholest-7-en-3β-ol; $\Delta^{8(14)}$-cholestenol, 5α-cholest-8(14)-en-3β-ol; $\Delta^{7,14}$-cholestadienol, 5α-cholesta-7,14-dien-3β-ol; lophenol (or 4α-methyl-Δ^7-cholestenol), 4α-methyl-5α-cholest-7-en-3β-ol; 4,4-dimethyl-$\Delta^{7,9(11)}$-cholestadienol, 4,4-dimethyl-5α-cholesta-7,9(11)-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lanosterol, 5α-lanosta-8,24-dien-3β-ol; 24-methylenecholesterol, 24-methylenecholesta-5,24(28)-dien-3β-ol; 24-methylenecycloartanol, 24-methylene-9β,19-cyclo-5α-lanostan-3β-ol; *trans*-22-dehydrocholesterol, cholesta-5,E-22-dien-3β-ol; *cis*-22-dehydrocholesterol, cholesta-5,Z-22-dien-3β-ol; fucosterol, stigmasta-5,E-24(28)-dien-3β-ol; isofucosterol, stigmasta-5,Z-24(28)-dien-3β-ol; campestanol; (24*R*)-24-methyl-5α-cholestan-3β-ol; campesterol, (24*R*)-24-methylcholest-5-en-3β-ol; 22,23-dihydrobrassicasterol, (24*S*)-24-methylcholest-5-en-3β-ol; Δ^7-ergostenol, (24*S*)-24-methyl-5α-cholest-7-en-3β-ol; $\Delta^{8(14)}$-ergostenol, (24*S*)-24-methyl-5α-cholest-8(14)-en-3β-ol; stigmastanol, (24*R*)-24-ethyl-5α-cholestan-3β-ol; sitosterol, (24*R*)-24-ethylcholest-5-en-3β-ol; 4β-methylstigmastanol, 4β-methyl(24*R*)-24-ethyl-5α-cholestan-3β-ol; Δ^7-stigmastenol, (24*R*)-24-ethyl-5α-cholest-7-en-3β-ol; clionasterol, (24*S*)-24-ethylcholest-5-en-3β-ol; 22,23-dihydrochondrillasterol, (24*S*)-24-ethyl-5α-cholest-7-en-3β-ol; ergosterol, (24*R*)-24-methylcholesta-5,7,E-22-trien-3β-ol; $\Delta^{7,22}$-ergostadienol, (24*R*)-24-methyl-5α-cholesta-7,E-22-dien-3β-ol; brassicasterol, (24*R*)-24-methylcholesta-5,E-22-dien-3β-ol; stigmasterol, (24*S*)-24-ethylcholesta-5,E-22-dien-3β-ol; 4α-methylstigmasterol, 4α-methyl(24*S*)-24-ethylcholesta-5,E-22-dien-3β-ol; 4β-methylstigmasterol, 4β-methyl(24*S*)-24-ethylcholesta-5,E-22-dien-3β-ol; spinasterol, (24*S*)-24-ethyl-5α-cholesta-7,E-22-dien-3β-ol; poriferasterol, (24*R*)-24-ethylcholesta-5,E-22-dien-3β-ol; chondrillasterol, (24*R*)-24-ethyl-5α-cholesta-7,E-22-dien-3β-ol; 25(27)-dehydrospinasterol, (24*R*)-24-ethyl-5α-cholesta-7,E-22,25(27)-trien-3β-ol; 25(27)-dehydrochondrillasterol, (24*S*)-24-ethyl-5α-cholesta-7,E-22,25(27)-trien-3β-ol.

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substituent at C-17 (see **Fig. 1**). Although the problem has recently been solved partially by the use of 220, 270 and particularly 360 MHz ^{1}H-NMR with superconducting magnets (9–17), such instruments have not yet come into general use. In addition, the signals arising from complex molecules occasionally have overlapped even if such instruments were used. Accordingly, it is desirable to develop an efficient method

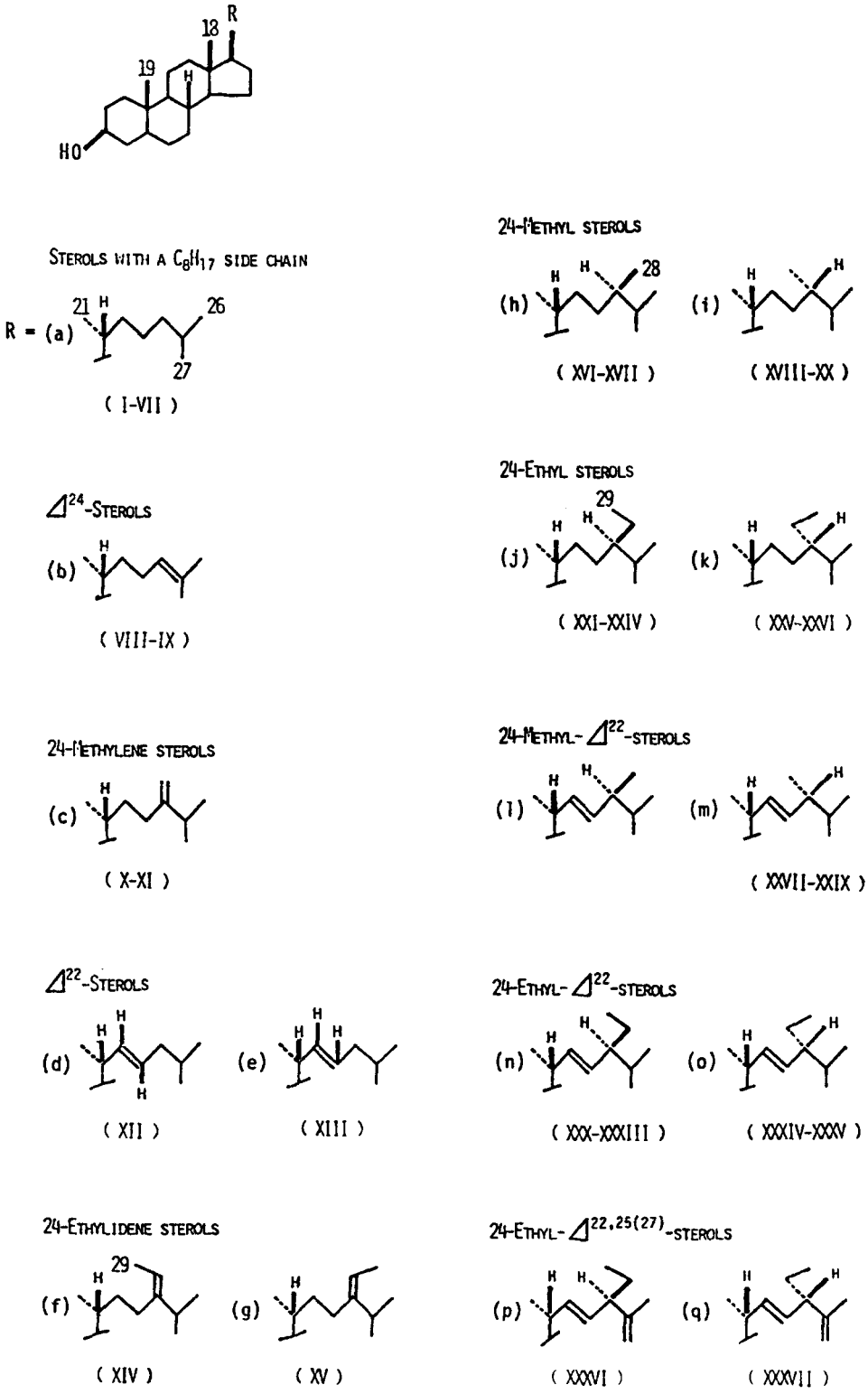

Fig. 1. Side chain structures of phytosterols.

for identifying the isomeric sterols by the most commonly used ^{1}H-NMR at 90 or 100 MHz.

In our previous studies on the 90 MHz ^{1}H-NMR studies, we reported on the effectiveness of a lanthanide shift reagent (LSR) (18, 19), e.g., tris(2,2,6,6-tetramethyl-3,5-heptanedionato)europium, Eu(dpm)$_3$, in structural and stereochemical problems of closely related compounds such as steroids and terpenoids (20–22). The present report describes the usefulness of the LSR for identifying a number of biologically important phytosterols with structurally different aliphatic side chains shown in Fig. 1, using a 90 MHz ^{1}H-NMR spectrometer. Tris[1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato]ytterbium, Yb(fod)$_3$, was employed in this study as the LSR, because of its strong coordinating ability with an oxygen-containing substrate and also its superior solubility in NMR solvents (e.g., $CDCl_3$) (23). As a result, the application of Yb(fod)$_3$ in ^{1}H-NMR (at 90 MHz) could characterize each of the isomeric sterols with different spatial configurations of double bonds and alkyl groups at C-17 in the side chain.

EXPERIMENTAL

Samples and reagents

The sources of sterols used in this study were as follows. Cholesterol and 24-methylenecycloartanol were from Riken Vitamin Oil Co., (Tokyo, Japan). Lanosterol was from Nakarai Chemicals Ltd., (Tokyo, Japan). Ergosterol was from Gasukuro Kogyo Works Ltd., (Tokyo, Japan). Desmosterol was from Steraloids Inc., (Wilton, NH). Campesterol, sitosterol, and stigmasterol were from Applied Science Laboratories Inc., (State College, PA).

The following samples were gifts from individuals: *trans*-22-dehydrocholesterol and its *cis*-isomer from Dr. M. J. Thompson, Insect Physiology Laboratory, U.S. Department of Agriculture; isofucosterol, 25(27)-dehydrospinasterol, and 25(27)-dehydrochondrillasterol from Dr. W. Sucrow, Lehrgebiet Organische Chemie, Gesamthochshule Paderborn; brassicasterol and its 22,23-dihydro derivative from Dr. H. W. Kircher, College of Agriculture, University of Arizona; 4β-methylstigmastanol, 4α-methylstigmasterol, and 4β-methylstigmasterol from Dr. G. J. Schroepfer, Jr., Department of Biochemistry, Rice University; clionasterol and poriferasterol from Dr. W. R. Nes, Department of Biological Science, Drexel University; poriferasterol from L. J. Goad, Department of Biochemistry, University of Liverpool; chondrillasterol and its 22,23-dihydro derivative from G. W. Patterson, Department of Botany, University of Maryland.

The preparation of cholestanol, lathosterol, lophenol, $\Delta^{8(14)}$-cholestenol, $\Delta^{7,14}$-cholestadienol, and 4,4-dimethyl-$\Delta^{7,9(11)}$-cholestadienol has been previously described (24). Campestanol and stigmastanol were prepared from campesterol and stigmasterol, respectively (25). $\Delta^{7,22}$-Ergostadienol was obtained by selective reduction of ergosterol (26). Δ^{7}-Ergostenol and $\Delta^{8(14)}$-ergostenol were derived from $\Delta^{7,22}$-ergostadienol (27). Fucosterol was isolated from brown algae (28). 24-Methylenecholesterol, Δ^{7}-stigmastenol, and spinasterol were isolated from akamegashiwa, pumpkin, and tea seed oils, respectively (29, 30).

Yb(fod)$_3$ as a LSR was available from E. Merck (Darmstadt, Germany); it was stored in vacuo over P_2O_5, and used without further purification.

Procedure

All the ^{1}H-NMR spectra were measured in $CDCl_3$ solutions with tetramethylsilane (TMS) as an internal reference standard, at an ambient probe temperature of 34°C; a Hitachi R-22 (90 MHz) spectrometer was used. Concentrations of the solutions were 0.2–3.2 $\times 10^{-5}$ M depending upon the sample quantity available. Each normal spectrum of pure substrates was first recorded at sweep width of 800 Hz, and subsequently the lanthanide-induced shift (LIS) spectra were obtained in the following manner. Exact amounts of Yb(fod)$_3$ were added in increasing amounts to the solutions of a known quantity of the substrates in $CDCl_3$, and the spectra were recorded after each addition; usually seven or eight such additions of the LSR were made for each sample within the molar ratio of the reagent to the substrates up to about 1.5. Chemical shift values were denoted in δ (ppm) relative to internal TMS and coupling constant (j) in Hz. Accuracy of the measurements was estimated to be less than 0.01 ppm for the chemical shifts.

After the ^{1}H-NMR measurement of Yb(fod)$_3$-substrate complexes in $CDCl_3$, Yb(fod)$_3$-free sterols were easily recovered as a pure state from the mixtures by a short column chromatography (8 cm $\times$ 0.8 mm i.d.) with ethyl ether–methyl alcohol (95:5, by vol.) as eluant and activated alumina (grade II, 200 mesh) as adsorbent. The recovered samples were checked by gas–liquid chromatography on a column of 1% OV-17 and ^{1}H-NMR spectroscopy.

RESULTS AND DISCUSSION

Normal ^{1}H-NMR spectra

The normal 90 MHz ^{1}H-NMR chemical shifts for methyl protons before adding LSR to the sterols examined in this study are shown in **Table 1.** Each methyl

TABLE 1. Normal 90 MHz ^{1}H-NMR chemical shifts for methyl protons in sterols examined

Compounds	Configuration at C-24[a]	Chemical Shifts (δ-ppm relative to TMS) C-18[b]	C-19[b]	C-21[c]	C-26/C-27[d]	C-28[e]	C-29[f]	Others[g]
Sterols with a C_8H_{17} side chain								
I. Cholestanol		0.65	0.81	0.89	0.87			
II. Cholesterol		0.70	1.02	0.92	0.87			
III. Lathosterol		0.54	0.80	0.91	0.87			
IV. $\Delta^{8(14)}$-Cholestenol		0.86	0.70	0.95	0.89			
V. $\Delta^{7,14}$-Cholestadienol		0.85	0.92	0.97	0.88			
VI. Lophenol		0.54	0.84	0.92	0.87			0.98[g1]
VII. 4,4-Dimethyl-$\Delta^{7,9(11)}$-cholestadienol		0.49	0.90	0.95	0.87			1.00,[g2] 0.97[g3]
Δ^{24}-Sterols								
VIII. Desmosterol		0.69	1.02	0.95	1.59/1.68			
IX. Lanosterol		0.70	1.01	0.91	1.59/1.68			0.98,[g2] 0.80,[g3] 0.87[g4]
24-Methylene sterols								
X. 24-Methylenecholesterol		0.70	1.02	0.98	1.03			
XI. 24-Methylenecycloartanol		0.99		0.91[h]	1.03			0.97,[g2] 0.81,[g3] 0.91[g4]
Δ^{22}-Sterols								
XII. *trans*-22-Dehydrocholesterol		0.70	1.02	1.01	0.86			
XIII. *cis*-22-Dehydrocholesterol		0.73	1.02	0.96	0.90			
24-Ethylidene sterols								
XIV. Fucosterol		0.70	1.04	0.99	1.00		1.58	
XV. Isofucosterol		0.70	1.04	0.96	0.99		1.59	
24-Methyl sterols								
XVI. Campestanol	24*R*/α-CH_3	0.66	0.81	0.89	0.85/0.80	0.80		
XVII. Campesterol	24*R*/α-CH_3	0.70	1.03	0.93	0.86/0.81	0.81		
XVIII. 22,23-Dihydrobrassicasterol	24*S*/β-CH_3	0.69	1.02	0.93	0.87/0.79	0.79		
XIX. Δ^7-Ergostenol	24*S*/β-CH_3	0.54	0.80	0.92	0.86/0.79	0.79		
XX. $\Delta^{8(14)}$-Ergostenol	24*S*/β-CH_3	0.85	0.70	0.93	0.86/0.79	0.79		
24-Ethyl sterols								
XXI. Stigmastanol	24*R*/α-C_2H_5	0.65	0.80	0.90	0.84/0.81		0.85	
XXII. Sitosterol	24*R*/α-C_2H_5	0.69	1.03	0.92	0.85/0.82		0.86	
XXIII. 4β-Methylstigmastanol	24*R*/α-C_2H_5	0.64	0.86	0.90	0.84/0.81		0.86	0.90[g5]
XXIV. Δ^7-Stigmastenol	24*R*/α-C_2H_5	0.54	0.81	0.92	0.85/0.82		0.86	
XXV. Clionasterol	24*S*/β-C_2H_5	0.69	1.03	0.93	0.84/0.82		0.87	
XXVI. 22,23-Dihydrochondrillasterol	24*S*/β-C_2H_5	0.54	0.81	0.91	0.84/0.82		0.87	
24-Methyl-Δ^{22}-sterols								
XXVII. Ergosterol	24*R*/β-CH_3	0.64	0.97	1.04	0.84/0.83	0.93		
XXVIII. $\Delta^{7,22}$-Ergostadienol	24*R*/β-CH_3	0.56	0.81	1.02	0.85/0.83	0.92		
XXIX. Brassicasterol	24*R*/β-CH_3	0.71	1.02	1.03	0.85/0.83	0.92		
24-Ethyl-Δ^{22}-sterols								
XXX. Stigmasterol	24*S*/α-C_2H_5	0.72	1.03	1.03	0.85/0.79		0.82	
XXXI. 4α-Methylstigmasterol	24*S*/α-C_2H_5	0.71	1.05	1.02	0.85/0.80		0.82	1.11[g1]
XXXII. 4β-Methylstigmasterol	24*S*/α-C_2H_5	0.70	1.06	1.02	0.85/0.80		0.82	1.14[g5]
XXXIII. Spinasterol	24*S*/α-C_2H_5	0.56	0.82	1.03	0.86/0.80		0.82	
XXXIV. Poriferasterol	24*R*/β-C_2H_5	0.71	1.02	1.03	0.85/0.79		0.82	
XXXV. Chondrillasterol	24*R*/β-C_2H_5	0.56	0.82	1.03	0.85/0.79		0.82	
24-Ethyl-$\Delta^{22,25(27)}$-sterols								
XXXVI. 25(27)-Dehydrospinasterol	24*R*/α-C_2H_5	0.55	0.81	1.02	1.65[b]/4.74[i]		0.83	
XXXVII. 25(27)-Dehydrochondrillasterol	24*S*/β-C_2H_5	0.56	0.82	1.02	1.66[b]/4.74[i]		0.84	

[a] Configuration at C-24 alkyl groups was symbolized by combined use of the (*R*, *S*)- and (α, β)-nomenclatures, as the introduction of a Δ^{22}- (or $\Delta^{25(27)}$-) bond reverses the specification of chirality at C-24 (44, 45).
[b] Singlet.
[c] Doublet (*j*, 6–7 Hz).
[d] Doublet (*j*, 6–7 Hz), except for compounds VIII and IX (see text).
[e] Doublet (*j*, 6–7 Hz).
[f] Triplet (*j*, 6–7 Hz), except for compounds XIV and XV in which the methyl signal occurred as doublet (*j*, 8 Hz).
[g] 1) C-4α (doublet, *j*, 6 Hz); 2) C-4α (singlet); 3) C-4β (singlet); 4) C-14α (singlet); 5) C-4β (doublet, *j*, 7 Hz).
[h] Estimated value by extrapolating method in the LIS spectra (see text).
[i] Methylene proton (broad singlet).

signal was almost exclusively assigned on the basis of the criteria discussed in the previous papers (4–14) of related compounds and also the comparison with the spectra of appropriate analogous derivatives. The assignments made were further confirmed from the LIS spectra (see below). As a result, the following gross features were inferred from the 90 MHz data.

a) The resonance position of singlets from the angular C-18 and C-19 methyl protons affords much useful information concerning the presence or absence and the position of substituents in the steroid nucleus. For example, the C-18 and C-19 methyls in compounds having Δ^5-bond in the nucleus occurred at 0.69–0.73 and 1.02–1.06 ppm, respectively, while the corresponding methyls were observed at 0.54–0.56 and 0.80–0.84 ppm, respectively, in compounds having a Δ^7-bond.

b) On the contrary, substituents in the side chain have little, if any, effect on the resonance position of the C-18 and C-19 methyls.

c) In sterols with a C_8H_{17}-saturated side chain (I–VII) and their 24-methylene (X–XI), Δ^{22}- (XII–XIII), and 24-ethylidene (XIV–XV) derivatives, the terminal C-26 and C-27 isopropyl methyl protons exhibit a magnetically equivalent doublet. However, the corresponding methyls in the other C-24 alkylated sterols (XVI–XXXV) are non-equivalent and give two sets of doublets. The phenomena result mainly from intrinsic magnetic non-equivalence generated by the C-24 chiral center (10). In this study the upper-field doublet of the two doublets from the terminal methyls in the C-24 alkylated sterols is tentatively assigned to the C-27 methyl proton and the lower-field doublet to the C-26 methyl proton, for the sake of ready comparison.

d) The difference in the chemical shifts between the C-26 and C-27 methyl signals in the C-24 alkylated sterols is influenced by the presence or absence of a Δ^{22}-bond and by the attribute of a C-24 alkyl group, e.g., difference in the polarizability of methyl and ethyl groups (10). In 24-methyl sterols (XVI–XX), the two doublets were more separated from each other than those in 24-methyl-Δ^{22}-sterols (XXVII–XXIX); however, the reverse relationship was observed between 24-ethyl (XXI–XXVI) and 24-ethyl-Δ^{22}-(XXX–XXXV) sterols.

e) Introduction of a Δ^{22}-bond causes significant downfield shifts (ca. 0.11 ppm) of the doublets due to the C-21 and C-28 methyl protons in the 24-methyl-Δ^{22}-sterols. A similar effect is also observed on the C-21 methyl proton resonance in the 24-ethyl-Δ^{22}-sterols.

f) A triplet occurring from the splitting of the C-29 methyl proton in the 24-ethyl sterols as well as their C-22 unsaturated analogs is not visualized distinctly, entirely or partially, due to the overlapping with other strong signals, with the exception of 24-ethyl-$\Delta^{22,25,(27)}$-sterols (XXXVI–XXXVII) which show a distinct triplet of the methyl by the influence of a $\Delta^{25(27)}$-bond.

Although the above observations seem to be useful as a measure for characterizing each series of sterols, the stereochemical identification of side chain substituents remains obscure. To solve this problem in an unambiguous way and also to obtain more detailed information concerning the side chain structures of the individual sterols, the 90 MHz ^{1}H-NMR spectra in the presence of various amounts of a LSR, Yb(fod)$_3$, were examined next.

Yb(fod)$_3$–LIS spectra

Previous papers on LIS studies have premised that a LSR coordinates solely with an oxygen-containing function such as a hydroxyl, carbonyl, or acetoxyl group, and not with a double bond or a hydrocarbon (18). According to the finding of Hinckley (31) in the Eu(dpm)$_3$ (dipyridine adduct) LIS spectrum of cholesterol, the LSR acted definitely, not only on protons in the immediate vicinity of the coordinating site (C-3β-hydroxyl oxygen atom) of the Eu^{3+} but also on the terminal C-26 and C-27 methyls located far apart (ca. 13 Å) from the coordinating site. Furthermore, we (20) and Romeo, Giannetto, and Aversa (32) have shown that the coordinated Eu^{3+} approaches the C-3β-hydroxyl oxygen atom from the direction of the C—O bond, being 3 Å apart from the C-3 oxygen atom with an Eu^{3+}-O-C (3) angle of 128–130°. We (23) have also demonstrated that the coordinating ability of Yb(fod)$_3$ is ca. three times as strong as that of Eu(dpm)$_3$. On the basis of these previous findings, the side chain methyls (C-21, C-26, C-27, C-28, and C-29) in sterols I–XXXVII would be expected to be affected by Yb(fod)$_3$. In fact, consistent, though very small, effects on the methyl protons were detected (see below).

Upon successive addition of Yb(fod)$_3$ to the sterol solutions in $CDCl_3$, all the substrate methyl protons suffered the expected shifts to downfield (23) without notable line broadening. The signal assignment of the LIS spectra was made from a knowledge of the relative intensities, signal multiplicity (singlet, doublet or triplet) observed, and the measurement of an approximate spatial distance between the C-3 oxygen atom and the methyls under consideration. The magnitude of coupling constants was virtually unaffected under the conditions described in the Experimental section. Although the resonance peaks due to the *t*-butyl group in Yb(fod)$_3$ appeared at ca. 2 and 4 ppm, respectively, with increasing amounts of the LSR, the occurrence

of the peaks was not a serious problem. The spectra of *cis*-22-dehydrocholesterol (XIII) with and without the LSR are illustrated in **Fig. 2.**

Plots of the chemical shifts of the methyl protons versus the molar ratio of $Yb(fod)_3$ added to the substrates showed a good linearity, particularly concern-

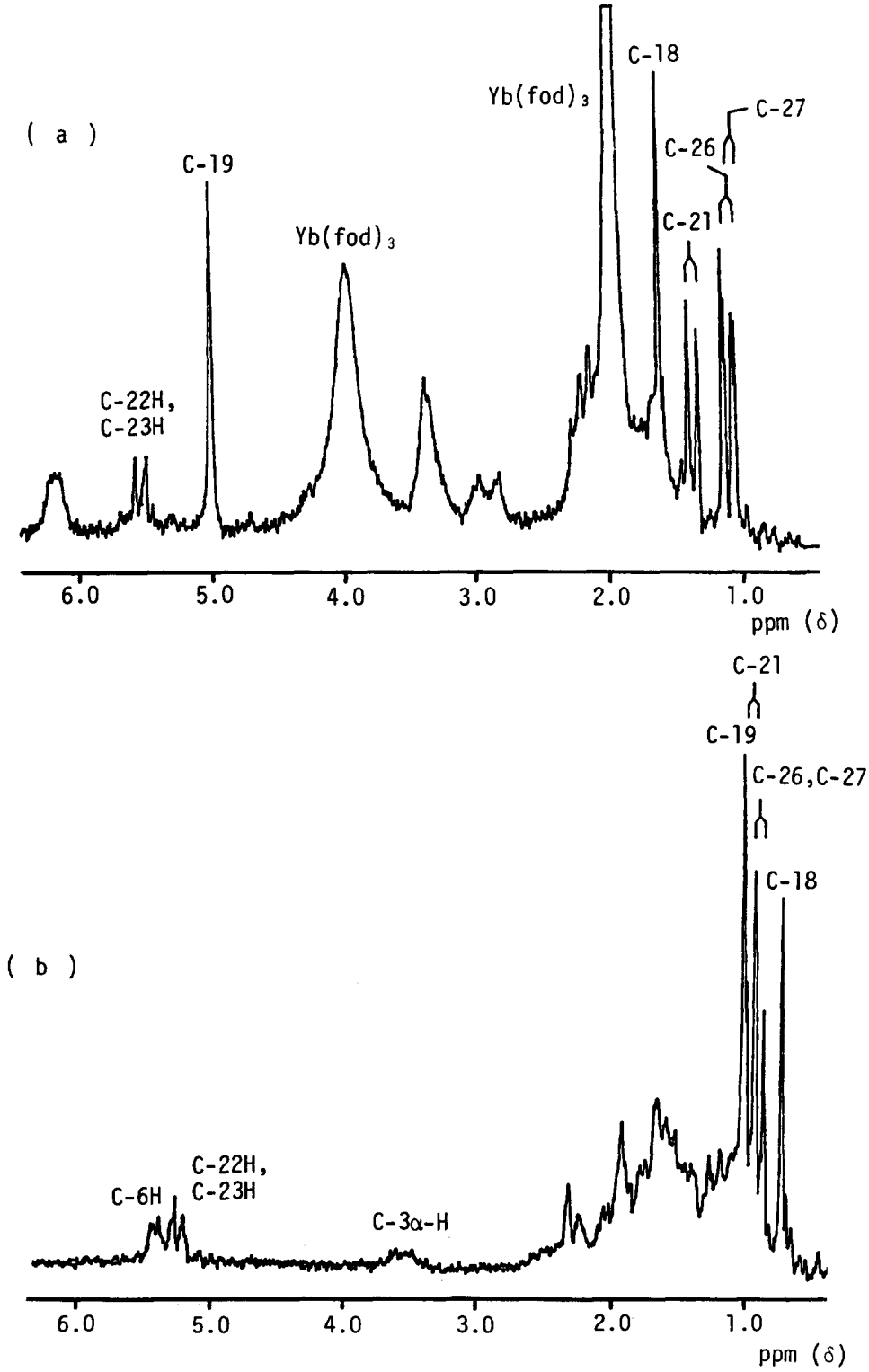

Fig. 2. 90 MHz ^{1}H-NMR spectra of *cis*-22-dehydrocholesterol (XIII, 4.0 mg), (a) with and (b) without 1.2 mol equivalent of $Yb(fod)_3$ in $CDCl_3$.

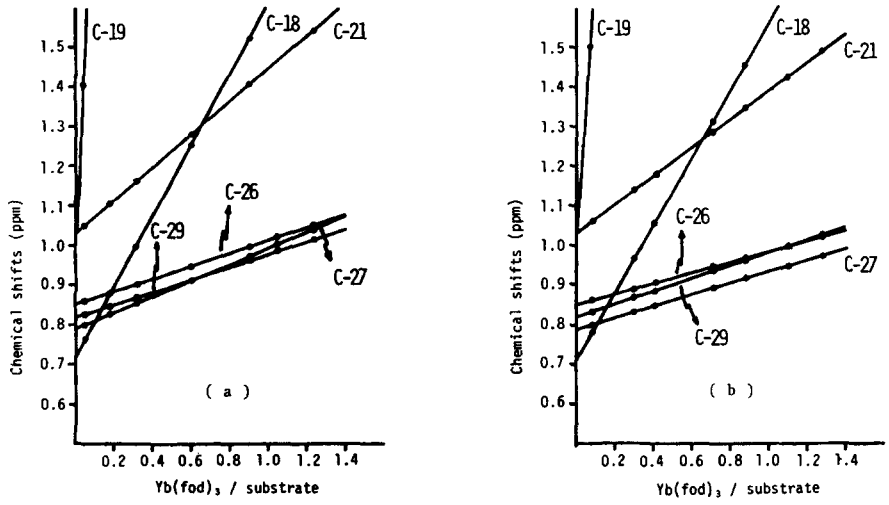

Fig. 3. Variation in the chemical shifts for the methyl protons of (a) stigmasterol (XXX: 1.8×10^{-5} M) and (b) poriferasterol (XXXIV; 0.7×10^{-5} M) with increasing concentration of $Yb(fod)_3$.

ing side chain methyl plots, within the molar ratio up to ca. 1.5. This observation indicates that tracing a particular signal back to the ordinate enables verification of the assignment from a knowledge of the initial chemical shift of the particular resonance in the absence of added LSR. In **Fig. 3,** sample plots in stigmasterol (XXX) and its C-24 epimer, poriferasterol (XXXIV), are dipicted. A similar linearity has also been found on the methyl plots of C-4-methylated steroids (20), triterpenes related to β-amyrin (33, 34) and 12-ursene (32). As can be seen in Figs. 2 and 3, the side chain methyls were shifted to a smaller extent on aliquot addition of $Yb(fod)_3$, compared with the skeletal C-18 and C-19 methyls which could easily be assigned. However, a careful inspection of the LIS curves provided valuable information about the stereoisomeric configuration of the methyls or the geometry of double bonds in the side chain.

Thus, the induced chemical shifts (ppm) for the side chain methyls were determined from the plotting data by the equation defined as $\delta_{n=1}{}^{Yb(fod)_3} - \delta_{n=0}$, where $\delta_{n=1}{}^{Yb(fod)_3}$ is the chemical shift obtained by extrapolating to the point where the molar ratio is 1.0, and $\delta_{n=0}$ is the chemical shift at zero concentration of $Yb(fod)_3$. Because of the dependence of the magnitude of the induced chemical shifts on the substrate concentration (22), and in order to minimize the effect of experimental error in both the LSR and substrate concentrations, the observed values for the fastest moving side chain methyl signal were further normalized to a value of 1.0, and the normalized values were expressed in terms of the induced shift ratios (ISR values). This normalization procedure is essentially the same as normalized chemical shifts (35), $\delta_{ind.}$ values (32), and shift attenuation factors (36), respectively, proposed by previous workers.

Next, in order to confirm the reliability and reproducibility of the ISR values, the LIS spectra were measured with several different concentrations of the same substrate (I, VII, XIV, XXII, XXVII, and XXX). As expected, the observed ISR values for the corresponding protons were almost independent of the substrate concentrations, showing at most a slight deviation of the values of ca. 0.03. Therefore, in the compounds having identical side chain structures, the possible origin of a deviation more than the value of ca. 0.05 may be attributed to the difference of the ring structures. These LIS data are summarized in **Table 2.**

It is evident from Table 2 that the ISR values for the side chain methyls are sensitive to both the stereochemical nature and the chemical environments about the methyls, but usually insensitive to the ring structures with a few exceptions (see below): the ISR values for the corresponding signals in compounds having the same side chain structure were extremely similar to each other.

Sterols with a C_8H_{17} side chain

Among the signals from the side chain methyls of all of the compounds examined here, the doublet from the C-21 methyl proton was shifted to the largest extent by adding $Yb(fod)_3$, in accordance with its relative proximity to the coordinating site. Hence, in sterols (I–VII) with a C_8H_{17}-saturated side chain, the doublet, often overlapped with other strong signals in the normal 90 MHz spectra, was unambiguously discerned. The doublet from the equivalent C-26 and

C-27 isopropyl methyls in these compounds showed somewhat different ISR values, suggesting the influence of nuclear substituents, particularly of a C-4, 4-*gem*-dimethyl group adjacent to the coordinating site (compound VII).

Δ^{24}-Sterols

The introduction of a Δ^{24}-bond (compounds VIII and IX) caused a marked downfield shift in the resonance frequencies of the terminal C-26 and C-27 isopropylidene methyls (37); the upper-field signal at 1.59 ppm is attributable to the C-26 methyl *trans* to the C-24 hydrogen and the lower-field signal at 1.68 ppm is then attributable to the C-27 methyl (38). The effect of $Yb(fod)_3$ on the methyls appears to be slightly larger for the *trans*-methyl than for the *cis*-counterpart. A similar result was more easily observed in the LIS spectra of the acetate derivatives by using $Ho(fod)_3$ instead of $Yb(fod)_3$ as a LSR.[2] Again, the ISR values for the methyls in IX were appreciably larger than those in VIII, probably due to the presence of a C-4, 4-*gem*-dimethyl group.

24-Methylene sterols

In the normal 90 MHz spectra of 24-methylene sterols (X and XI), the doublet signals arising from the C-21 methyl were obscured due to the overlapping of proton signals from the different methyls. Under the influence of $Yb(fod)_3$, the signals were sufficiently displaced from the other signals so that unambiguous assignments could be made. However, a more diagnostic feature in the LIS spectra was the appearance of the two sets of doublets for the terminal C-26 and C-27 isopropyl methyls that were now rendered nonequivalent.

Δ^{22}-Sterols

It has already been stated (39) that the *cis*- and *trans*-geometrical isomers of Δ^{22}-sterols (XIII versus XII) are distinguishable from each other by a small difference of chemical shift in the resonance position of the C-18 and C-21 methyls, even though their spectra are recorded at 100 MHz in the absence of $Yb(fod)_3$. However, a more marked difference serving to differentiate between the two isomers was observed when the 90 MHz spectra were measured in the presence of the LSR. Thus, while the *trans*-isomer showed an expected doublet for equivalent C-26 and C-27 isopropyl methyls, the corresponding methyls in the *cis*-isomer were resolved into the two sets of doublets (see Fig. 2). The signal resolution of the methyls in the *cis*-Δ^{22}-sterol was much more prominent than that of the 24-methylene sterols mentioned above and may be ascribed to the restriction of free rotation about the C-24/C-25 bond (by using a Dreiding model). Furthermore, the ISR values for the methyls were found to be larger in the *cis*-isomer than in the *trans*.

24-Ethylidene sterols

Differentiation between the *cis*- and *trans*-isomers of 24-ethylidene sterols, i.e., fucosterol (XIV) versus isofucosterol (XV), could be made even at their normal 90 MHz spectra by the chemical shift difference in the heptet arising from the C-25 isopropyl metinyl proton (*cis*, 2.3 ppm; *trans*, 2.8 ppm) and the C-21 methyl (Table 1) (10, 40, 41). The two isomers were further characterized by the fact that, in the *cis*-isomer, $Yb(fod)_3$ induces a slightly larger shift on the C-29 methyl than the C-26 and C-27 isopropyl methyls, while, in the *trans*-isomer, the LSR affects the three methyls to a similar extent. These LIS behaviors are in accord with approximate distance of those methyls from the coordinating site. Again, the presence of the C-21 methyl signals, which are overlapped with the C-26 and C-27 methyl signals in the normal 90 MHz spectra, was unambiguously confirmed by adding $Yb(fod)_3$.

24-Methyl sterols

The $Yb(fod)_3$-LIS spectra of 24*R*/α- (XVI–XVII) and 24*S*/β- (XVIII–XX) methyl sterols showed a marked difference in the induced shifts of the C-26, C-27, and C-28 methyls. In the 24*R*/α-methyl sterols, three doublets arising from the C-26, C-27, and C-28 methyls had the same ISR values. On the other hand, the corresponding values in the 24*S*/β-methyl sterols were consistently larger for the C-28 methyl than for the C-26 and C-27 methyls that were shifted to a similar extent. Since C-24 epimeric pairs of 24-methyl sterols, e.g., campesterol (XVII) versus 22,23-dihydrobrassicasterol (XVIII), generally have very similar chemical shifts for all of the methyl protons in the normal 90 MHz spectra, the above finding can therefore be used as a diagnostic tool to distinguish between the C-24 epimeric 24-methyl sterols.

24-Ethyl sterols

No clear feature serving to characterize the configuration at C-24 of 24*R*/α- (XXI–XXIV) and 24*S*/β- (XXV–XXVI) ethyl sterols could also be detected in their normal spectra recorded at 90 MHz. Under the influence of $Yb(fod)_3$, the C-26, C-27, and C-29 methyls in these compounds were shifted to the same

[2] Iida, T., T. Tamura, and T. Matsumoto. Unpublished experiments.

TABLE 2. $Yb(fod)_3$-LIS data for methyl protons in sterols examined

Compounds	Concentration of Substrates ($\times 10^{-5}$ M)	Induced Chemical Shifts (ppm)[a]						ISR Values for Side Chain Methyl Protons[b]			
		C-18	C-19	C-21	C-26/C-27	C-28	C-29	C-21	C-26/C-27	C-28	C-29
Sterols with a C_8H_{17} side chain											
I	0.8	0.85	3.60	0.36	0.12			1.00	0.33		
	2.6	1.05	5.05	0.50	0.17			1.00	0.34		
	3.2	1.25	5.85	0.66	0.21			1.00	0.32		
II	2.0	0.90	4.30	0.44	0.14			1.00	0.32		
III	2.0	0.95	4.10	0.46	0.16			1.00	0.35		
IV	2.4	1.10	4.85	0.50	0.17			1.00	0.34		
V	2.9	1.20	5.75	0.46	0.15			1.00	0.33		
VI	2.2	1.05	4.45	0.50	0.19			1.00	0.38		
VII	2.5	1.15	4.35	0.40	0.18			1.00	0.45		
	3.0	1.25	4.85	0.44	0.19			1.00	0.43		
Δ^{24}-Sterols											
VIII	1.2	0.80	3.50	0.34	0.15/0.13			1.00	0.44/0.38		
IX	1.7	0.95	3.50	0.30	0.18/0.16			1.00	0.60/0.53		
24-Methylene sterols											
X	1.3	0.90	4.10	0.42	0.16/0.15			1.00	0.38/0.36		
XI	0.8	0.65		0.30	0.14/0.13			1.00	0.47/0.43		
Δ^{22}-Sterols											
XII	1.4	1.00	4.35	0.47	0.19			1.00	0.40		
XIII	1.0	0.80	3.45	0.36	0.19/0.17			1.00	0.53/0.47		
24-Ethylidene sterols											
XIV	0.7	0.65	2.70	0.29	0.10		0.13	1.00	0.34		0.45
	1.2	0.70	3.00	0.32	0.11		0.14	1.00	0.34		0.44
XV	0.3	0.50	2.15	0.24	0.08		0.08	1.00	0.33		0.33
24-Methyl sterols											
XVI	2.8	1.25	5.95	0.61	0.21/0.21	0.21		1.00	0.34/0.34	0.34	
XVII	2.1	1.00	4.75	0.50	0.17/0.17	0.17		1.00	0.34/0.34	0.34	
XVIII	1.5	0.90	4.15	0.42	0.13/0.13	0.16		1.00	0.31/0.31	0.37	
XIX	3.1	1.30	5.40	0.61	0.19/0.19	0.22		1.00	0.31/0.31	0.36	
XX	2.9	1.25	5.35	0.60	0.20/0.20	0.23		1.00	0.33/0.33	0.38	
24-Ethyl sterols											
XXI	2.6	1.35	5.80	0.61	0.21/0.21		0.21	1.00	0.34/0.34		0.34
XXII	1.1	0.80	3.40	0.36	0.12/0.12		0.12	1.00	0.33/0.33		0.33
	1.9	0.95	4.05	0.50	0.16/0.16		0.16	1.00	0.32/0.32		0.32
XXIII	0.4	0.90	3.50	0.38	0.12/0.12		0.12	1.00	0.32/0.32		0.32
XXIV	1.0	1.25	4.95	0.54	0.19/0.19		0.19	1.00	0.35/0.35		0.35
XXV	0.7	0.75	3.20	0.35	0.12/0.12		0.12	1.00	0.34/0.34		0.34
XXVI	0.9	1.15	4.85	0.58	0.19/0.19		0.19	1.00	0.33/0.33		0.33
24-Methyl-Δ^{22}-sterols											
XXVII	0.8	0.75	3.30	0.35	0.12/0.12		0.14	1.00	0.34/0.34		0.40
	1.3	0.85	3.75	0.37	0.13/0.13		0.15	1.00	0.35/0.35		0.41
	1.9	0.95	3.95	0.38	0.13/0.13		0.15	1.00	0.34/0.34		0.39
XVIII	3.2	1.35	5.95	0.63	0.21/0.21		0.26	1.00	0.33/0.33		0.41
XXIX	1.1	0.70	3.00	0.30	0.11/0.11		0.13	1.00	0.36/0.36		0.43
24-Ethyl-Δ^{22}-sterols											
XXX	1.8	0.95	4.15	0.42	0.16/0.20		0.16	1.00	0.38/0.48		0.38
	3.1	1.15	5.25	0.58	0.23/0.26		0.23	1.00	0.40/0.45		0.40
XXXI	0.2	0.40	1.55	0.23	0.09/0.11		0.09	1.00	0.39/0.48		0.39
XXXII	0.7	0.70	2.95	0.38	0.15/0.18		0.15	1.00	0.39/0.47		0.39
XXXIII	1.1	1.20	4.90	0.52	0.20/0.24		0.20	1.00	0.38/0.46		0.38
XXXIV	0.7	0.80	3.65	0.36	0.13/0.15		0.15	1.00	0.36/0.42		0.42
XXXV	0.9	1.00	4.20	0.40	0.14/0.17		0.17	1.00	0.35/0.43		0.43

TABLE 2. (*Continued*)

Compounds	Concentration of Substrates ($\times 10^{-5}$ M)	Induced Chemical Shifts (ppm)[a]						ISR Values for Side Chain Methyl Protons[b]			
		C-18	C-19	C-21	C-26/C-27	C-28	C-29	C-21	C-26/C-27	C-28	C-29
24-Ethyl-$\Delta^{22,25(27)}$-sterols											
XXXVI	1.1	0.95	3.85	0.41	0.17/0.16		0.16	1.00	0.41/0.39		0.39
XXXVII	1.4	1.05	4.35	0.47	0.18/0.18		0.18	1.00	0.38/0.38		0.38

[a] The values were obtained from the plotting data (see Fig. 3) by the equation defined as $\delta_{n=1}^{Yb(fod)_3} - \delta_{n=0}$, where $\delta_{n=1}^{Yb(fod)_3}$ is the chemical shift obtained by extrapolating to the point where the molar ratio is 1.0 and $\delta_{n=0}$ is the chemical shift at zero concentration of $Yb(fod)_3$.

[b] The values were defined as the ratios of the induced chemical shifts of the respective side chain methyls to that of the fastest moving side chain methyl. In all cases examined, the doublet signal due to the C-21 methyl was chosen as the reference signal.

extent. However, the removal of other methyl and methylene proton signals from the C-26, C-27, and C-29 methyl region by $Yb(fod)_3$ revealed a significant difference in the spectral patterns to permit the unambiguous assignment of the configuration at C-24 (see **Figs. 4a** and **4b**): in the 24*R*/α-ethyl sterols, two upper-field signals from parts of the triplet of the C-29 methyl coincide with the doublet signals from the C-27 methyl; in the 24*S*/β-ethyl sterols, the corresponding two signals from the C-29 methyl coincide with the

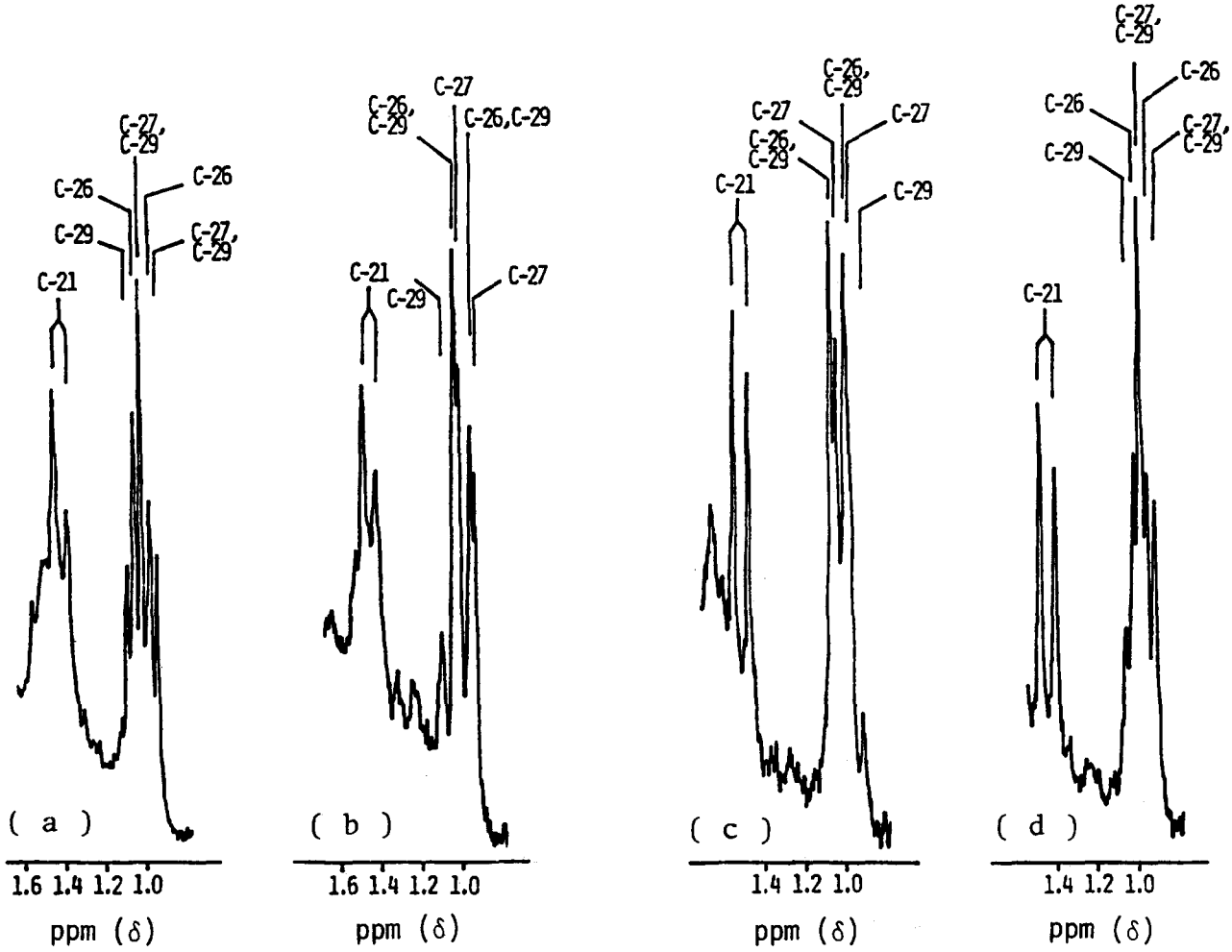

Fig. 4. 90 MHz ^{1}H-NMR spectra in the high field side chain methyl region of C-24 epimeric pairs of 24-ethyl and 24-ethyl-Δ^{22}-sterols containing an equivalent amount of $Yb(fod)_3$ in $CDCl_3$: (a) sitosterol (XXII, L/S = 1.6) and (b) clionasterol (XXV, L/S = 1.6); (c) stigmasterol (XXX, L/S = 1.2) and (d) poriferasterol (XXXIV), L/S = 1.2). L/S is the molar ratio of $Yb(fod)_3$ to the substrate.

doublet signals from the C-26 methyl. Such spectral patterns were virtually unaffected by the amount of Yb(fod)$_3$ added. By using the method, two C-24 epimeric pairs of 24-ethyl sterols, i.e., sitosterol (XXII) versus clionasterol (XXV), and Δ^7-stigmastenol (XXIV) versus 22,23-dihydrochondrillasterol (XXVI), could easily be differentiated from each other.

24-Methyl-Δ^{22}-sterols

In 24-methyl-Δ^{22}-sterols (XXVII–XXIX) with the R/β-configuration at C-24, the C-28 methyl on aliquot addition of Yb(fod)$_3$ moved somewhat faster than did both the C-26 and C-27 methyls. This LIS behavior closely resembled that observed for the corresponding C-22 saturated 24S/β-methyl sterols mentioned above. Therefore, the doublets due to the C-28 methyl and C-26 and C-27 isopropyl methyls were separated distinctly from each other. It should be noted here that, in the normal 90 MHz spectra of the 24R/β-methyl-Δ^{22}-sterols, the doublet signals from the C-27 methyl appear as a sparingly discernible shoulder on the doublet signals from the C-26 methyl, but the two doublets are overlapped by successive addition of Yb(fod)$_3$. This is presumably a result of a slight line broadening effect of the LSR.

24-Ethyl-Δ^{22}-sterols

Epimeric 24-ethyl-Δ^{22}-sterols with opposite configuration at C-24, i.e., stigmasterol (XXX) versus poriferasterol (XXXIV) and spinasterol (XXXIII) versus chondrillasterol (XXXV), also showed virtually identical normal 90 MHz spectra. The C-24 epimers, however, showed distinctly different Yb(fod)$_3$-LIS spectra and could easily be differentiated from each other. The difference was that the C-27 methyl in 24S/α-ethyl-Δ^{22}-sterols (XXX–XXXIII) has a somewhat larger ISR value than both the C-26 and C-29 methyls, while, in 24R/β-ethyl-Δ^{22}-sterols (XXXIV–XXXV), the C-26 methyl has a smaller value than both the C-27 and C-29 methyls (see Figs. 4c and 4d). As a result, the upper-field signal of the triplet from the C-29 methyl in the 24S/α-epimers was observed unequivocally without overlapping by other signals; on the other hand, the lower-field signal from the corresponding triplet was observed clearly in the LIS spectra of the 24R/β-epimers.

24-Ethyl-$\Delta^{22,25(27)}$-sterols

A C-24 epimeric pair of 24-ethyl-$\Delta^{22,25(27)}$-sterols (XXXVI versus XXXVII) was a sole example which could not be differentiated from each other in the presence of Yb(fod)$_3$. The two epimers must be differentiated by measuring the LIS spectra of their hydrogenated compounds in the side chain, which are readily derived by catalytic hydrogenation (14).

The above different effects of Yb(fod)$_3$ on the terminal C-26, C-27, and C-29 (or C-28) methyls in C-24 epimeric sterols are not interpreted simply based on the consideration of distance from the coordinating site; however, one of the possible factors may be related to the difference of the predominant conformers between C-20 and C-24 in the substituted side chains (42, 43).

In short, it may be seen from the foregoing that this study presents a useful and convenient method for the characterization of each member of a number of sterol pairs stereoisomeric in the side chain, using 90 MHz ^{1}H-NMR spectroscopy with the aid of a LSR, Yb(fod)$_3$. The method is essentially based on the difference in the Yb(fod)$_3$-LIS behaviors of the side chain methyls, which were generated by the stereochemical nature and the chemical environment of the methyl groups. By applying this procedure, we confirmed for the first time the presence of the C-24 epimer of spinasterol, i.e., chondrillasterol, in some species (*Cucurbitaceae* seed oils) of higher plants (46).

We are grateful to the individuals (see Samples and Reagents) for their generous gifts of sterol samples. Valuable comments on some of the problems by Dr. Y. Toyama are also gratefully acknowledged. This work has been supported in part by the Nihon University Scientific Research Subsidy.

Manuscript received 27 March 1979 and in revised form 9 October 1979.

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