Analysis of unsaturated C_{27} sterols by nuclear magnetic resonance spectroscopy

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Abstract Complete ^1H and ^{13}C nuclear magnetic resonance (NMR) signal assignments have been established for 5α-cholestan-3β-ol, 22 unsaturated C_{27} sterols, and their acetate derivatives. Assignments were made from a combination of 1D and 2D spectra and include stereochemical ^1H assignments for the C-22 and C-23 protons of 5α-cholesta-8,24-dien-3β-ol and other Δ^{24} sterols with a C_8 side chain. At the temperature and concentration range described, chemical shifts were generally reproducible to ±0.01 ppm in ^{13}C spectra and ±0.001 ppm in ^1H spectra. Except for some overlapped or strongly coupled ^1H resonances, chemical shifts are given to these precisions, which are an order of magnitude better than for most data given previously. Full ^1H NMR data have been reported previously for only three of the 46 compounds, and ^{13}C data were unavailable for many, including the previously undescribed cholesta-5,8(14)-dien-3β-ol. An extensive set of ^1H-^1H coupling constants for the unsaturated sterols indicated considerable conformational diversity, which was confirmed by molecular modeling. The conformational diversity together with other factors led to a complex pattern of ^{13}C substituent-induced chemical shifts (SCS) that appeared to elude any simple empirical correlations with structure. By contrast, the ^1H SCS correlated reasonably well with simple structural features. The high precision of the SCS revealed small but measurable effects of a double bond on ^1H resonances up to 12 bonds away. Also discussed are the utility and limitations of NMR for the identification of unsaturated sterols, estimation of purity, and analysis of mixtures, with an emphasis on special problems encountered at a microgram level.—**Wilson, W. K., R. M. Sumpter, J. J. Warren, P. S. Rogers, B. Ruan, and G. J. Schroepfer, Jr.** Analysis of unsaturated C_{27} sterols by nuclear magnetic resonance spectroscopy. *J. Lipid Res.* 1996. **37:** 1529–1555.

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The enzymatic formation of cholesterol from lanosterol involves a very large number of potential intermediates (1). The isolation and characterization of these sterol intermediates is complicated by their occurrence at only very low levels in blood and in most tissues. Rigorous identification of these sterols is frequently limited by the unavailability of reliable information concerning their chromatographic and spectral properties and/or the lack of appropriate standards of defined chemical structure.

These matters are of importance not only in the definition of the chemical nature of sterol intermediates in the biosynthesis of cholesterol but they are also of considerable potential import in biology and medicine. For example, the sterol composition of cells (and subcellular fractions derived therefrom) and culture media are of interest with regard to intracellular transport and efflux of sterols and cell morphology and function (2–5). Further interest in sterol intermediates has been stimulated by reports (6, 7) that the Smith-Lemli-Opitz syndrome, a severe genetic disorder affecting the normal development of multiple organ systems, is associated with the significant accumulation of several C_{27} noncholesterol sterols in blood and tissues. The biochemical defect in this disorder appears to be at the level of the conversion of 7-dehydrocholesterol to cholesterol (6–8). Additional impetus for research in the area of sterol intermediates in the biosynthesis of cholesterol derives from the recent report that certain di- and tri-unsatu-

Abbreviations and trivial names: COSYDEC, f_1-decoupled ^1H-^1H correlation spectroscopy; COSY-DQF, ^1H-^1H correlation spectroscopy with double-quantum filtering; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; DEPT, distortionless enhancement by polarization transfer; desmosterol, cholesta-5,24-dien-3β-ol; GC, gas chromatography; HETCOR, ^1H-^{13}C shift-correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; HSQC, heteronuclear single-quantum coherence; IR, infrared (spectroscopy); mp, melting point; MPLC, medium pressure liquid chromatography; MS, mass spectrometry or mass spectrum; NMR, nuclear magnetic resonance (spectroscopy); NOE, nuclear Overhauser enhancement; SC, side chain; SCS, substituent-induced chemical shifts; TLC, thin-layer chromatography; UV, ultraviolet (spectroscopy); vitamin D_3, (3S, 5Z, 7E)-9,10-secocholesta-5,7,10(19)-trien-3-ol; zymosterol, 5α-cholesta-8,24-dien-3β-ol.

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rated sterol intermediates appear to activate meiosis in mammalian oocytes (9).

Nuclear magnetic resonance (NMR) spectroscopy is an extremely powerful technique for the identification of sterols (10). To facilitate research in these areas, we have prepared a number of C_{27} sterols and present complete ^1H and ^{13}C NMR assignments for 5α-cholestan-3β-ol, 22 unsaturated C_{27} sterols, and their acetate derivatives. The breadth of compounds studied and the extraordinarily high precision of these data should advance the utility of NMR in the identification of unsaturated sterols.

EXPERIMENTAL PROCEDURES

Materials and methods

Melting points (mp) were measured with a Thomas-Hoover apparatus in sealed, evacuated capillary tubes. Infrared (IR) spectra were obtained from KBr pellets on a Mattson Galaxy 6020 Fourier-transform IR spectrometer. Low-resolution and high-resolution mass spectra (MS) were recorded on a VG ZAB-HF double-sector instrument with an electron energy of 70 eV after capillary gas chromatography (GC) or direct inlet sample introduction. Mass spectral data are given as *m/z* (relative abundance, suggested assignment). Relative abundances (for $m/z \geq 100$) are from low-resolution spectra, and exact masses are from high-resolution data. Exact masses are reported as the average of ~5 scans; standard deviations were typically 1–1.5 mmu for ions of ≥10% relative abundance.

Thin-layer chromatography (TLC) was carried out on silica gel G plates (Analtech; Newark, DE) or aluminum-backed silica gel 60 plates (EM Science; Gibbstown, NJ). Components on the plates were visualized after spraying with 5% ammonium molybdate(VI) in 10% sulfuric acid followed by heating. High performance liquid chromatography (HPLC) was carried out at a flow rate of 1 ml per min using a 5-μm Customsil ODS reversed phase column (250 mm × 4.6 mm i.d.; Custom LC; Houston, TX) and UV detection at 210 nm. Semi-preparative HPLC was done analogously using a 250 mm × 9.4 mm i.d. column at 3 ml per min. Solvent systems for TLC or HPLC were ethyl acetate–hexane 1:19 (SS-1); ethyl acetate–hexane 1:9 (SS-2); ethyl acetate–hexane 1:3 (SS-3); ethyl acetate–toluene 1:9 (SS-4); toluene–hexane 1:9 (SS-5); acetone–hexane 1:4 (SS-6); methanol–water 98:2 (SS-7). Column chromatography was performed on silica gel (70–230 mesh), and medium pressure liquid chromatography (MPLC) was done with a Lobar silica column (440 × 37 mm i.d.; EM Science) or a glass column dry-packed with silica gel (230–400 mesh). Fraction volumes were 20 ml. The adsorption of samples onto silica gel (11) and the preparation of silica gel-$AgNO_3$ (11) and alumina-$AgNO_3$ (12) have been described previously. Capillary GC was carried out isothermally at 250°C using split injection (9:1), helium or nitrogen carrier gas, and a 30-m or 60-m DB-5 column (0.25 mm i.d.; 5% phenyl, 95% methyl polysiloxane; 0.1 μm film thickness; J & W Scientific, Inc.; Folsom, CA).

Nuclear magnetic resonance (NMR) experiments were done on a Bruker AMX500 spectrometer (500.1 MHz for ^1H) equipped with a 5-mm inverse-geometry broadband probe and a 16-bit digitizer. ^1H NMR spectra were measured at 25°C in $CDCl_3$ solution (generally 5 to 20 mM) and referenced to internal tetramethylsilane or, for very dilute samples, $CHCl_3$ at δ 7.261. ^{13}C NMR spectra were measured at 22°C in $CDCl_3$ solution (5 to 150 mM) and referenced to $CDCl_3$ at 77.0 ppm. Probe temperatures designated as 22°C and 25°C corresponded to 1.537-ppm and 1.505-ppm ^1H chemical shift differences for a solution of 80% ethylene glycol in dimethylsulfoxide-d_6. NMR samples (0.5 ml in 507-PP or 528-PP tubes from Wilmad Glass Co.; Buena, NJ) were shimmed with spinning at 16–20 rps to give a narrow ($W_{1/2} \leq 0.5$ Hz), symmetrical line for tetramethylsilane; lines were somewhat broader for non-spinning experiments and samples of low-volume (0.35 ml $CDCl_3$). $CDCl_3$ (Cambridge Isotope Laboratories; Andover, MA) was either 99.8% D from screw-cap bottles or 99.96% D from sealed ampules. The latter solvent (purchased also from Aldrich Chemical Co.; Milwaukee, WI) was stored in the dark at 4°C.

Standard Bruker software was used to acquire DEPT (distortionless enhancement by polarization transfer), HETCOR (^1H-^{13}C shift-correlated spectroscopy), COSY-DEC (f_1-decoupled ^1H-^1H correlation spectroscopy), COSY-DQF (^1H-^1H correlation spectroscopy with double-quantum filtering), and HMBC (heteronuclear multiple bond correlation) spectra. The following parameters for COSYDEC spectra (13, 14) are representative: τ_c 0.2 s; 60° read pulse; 0.3-s acquisition time; 1-s relaxation delay; receiver gain half that used for the 1D proton spectrum; 256 t_1 increments of 4 scans each; f_2 window ca. δ -0.2 to 7.4; f_1 window ca. δ 0.5 to 2.5 (just encompassing the upfield protons); 11° shifted sine bell apodization in both dimensions; Fourier transform (magnitude calculation) with 2k points in t_2 and 512 points in t_1. The carrier frequency was adjusted so that the upfield region was folded exactly once in f_1. Parameters for the COSY-DQF experiment on **20a** were: 1.5-s acquisition time; 1-s relaxation delay; 512 t_1 increments of 8 scans each; f_2 window δ -0.4 to 3.2; f_1 window δ 0.5 to 2.3; phase-sensitive mode using the method of States, Haberkorn, and Ruben (15). Typical parameters for HMBC spectra were: 0.3-s acquisition time, 1.5-s relaxa-

tion delay; 3.85-ms delay (1/2J); 60-ms delay for evolution of long-range couplings; 100–200 t_1 increments of 32–96 scans each; f_2 window ca. δ_H - 0.2 to 7.4; f_1 window ca. δ_C 10 to 150; 90° shifted sine bell apodization in both dimensions; Fourier transform (magnitude calculation) with 2k points in t_2 and 256 points in t_1.

HSQC (heteronuclear single-quantum coherence) experiments were acquired with the standard pulse se-

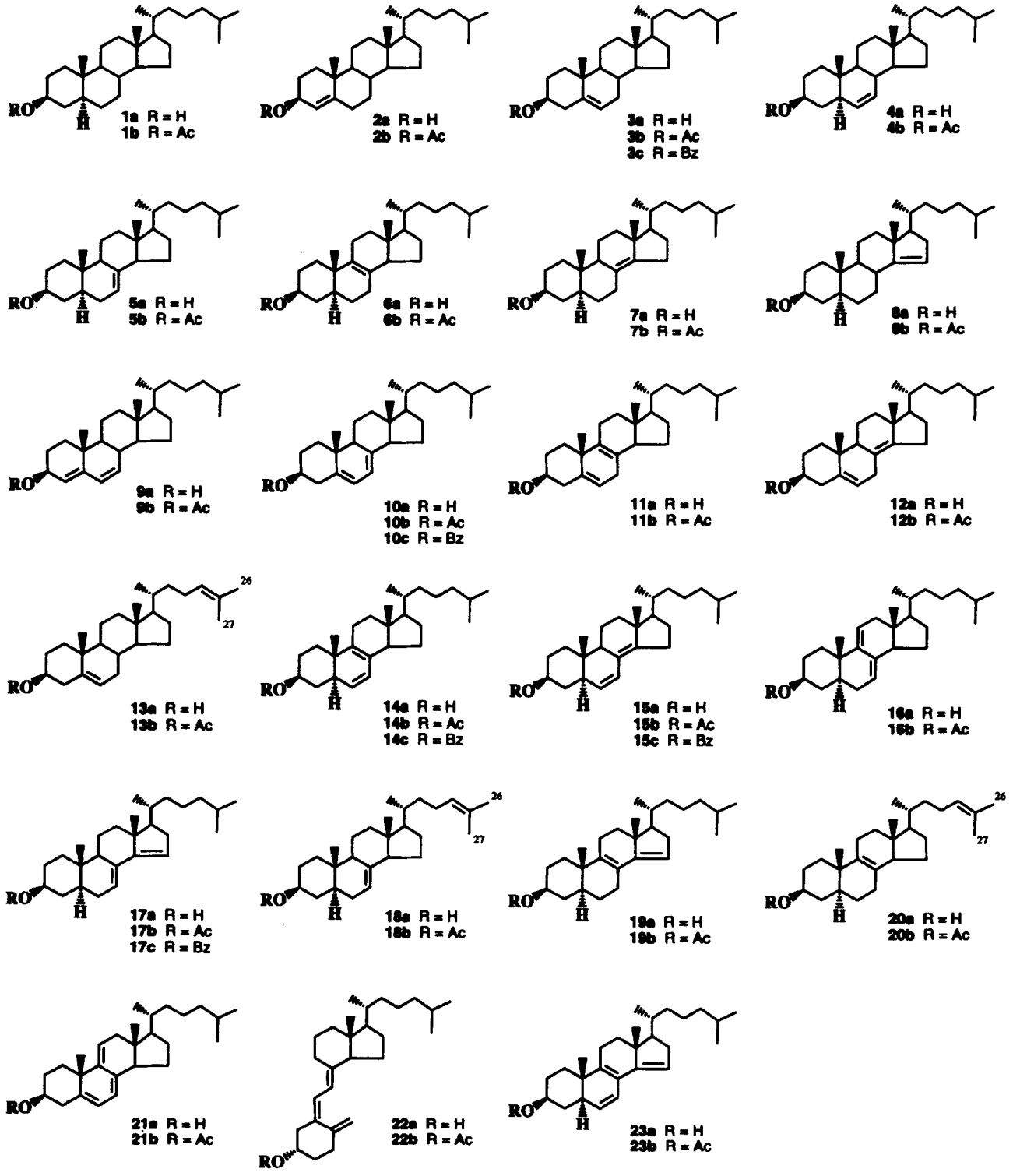

Fig. 1. Structures of 5α-cholestan-3β-ol and the unsaturated C_{27} sterols described herein.

quence (16) except for a 32-step phase cycle, replacement of the final 180° ^{13}C pulse by a composite pulse (90°–180°–90°), and phase incrementation by the States-TPPI method (17). Typical parameters were: 0.5-s acquisition time; 1.92-ms delay (1/4J); 1-s relaxation delay; GARP decoupling at minimal power (175 μs 90° pulse); 100 t_1 increments of 32 scans each; f_2 window ca. δ_H 0.5 to 2.5 (just encompassing the upfield protons); f_1 win-

Table 1. ^1H NMR chemical shifts of monoene free sterols **1a-8a** and acetates **1b-8b**[a]

	Δ^0		Δ^4		Δ^5		Δ^6		Δ^7		Δ^8		$\Delta^{8(14)}$		Δ^{14}	
	1a	**1b**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**	**7a**	**7b**	**8a**	**8b**
H-1α	0.961	1.008	1.259	1.319	1.077	1.132	1.043	1.082	1.081†	1.132	1.215	1.248	1.098	1.149	0.954	1.001
H-1β	1.707	1.725	1.693	1.720	1.844†	1.859	1.618	1.632†	1.823†	1.838†	1.756	1.773	1.695	1.714	1.774	1.791†
H-2α	1.794	1.800	1.949	1.960	1.834†	1.857†	1.866	1.871	1.80	1.815†	1.841	1.853	1.820	1.828	1.798	1.807†
H-2β	1.397	1.489	1.428	1.556	1.500	1.585	1.531	1.612†	1.389	1.475	1.441	1.525	1.356	1.449	1.413	1.502
H-3α	3.587	4.683	4.150	5.227†	3.524	4.603†	3.645	4.735	3.597	4.694	3.617	4.700	3.621	4.715	3.594	4.685
H-4α	1.553	1.586	5.272	5.217†	2.293	2.317†	1.691	1.727	1.717	1.740	1.645	1.684	1.628	1.659	1.586	1.621
H-4β	1.282	1.352			2.236	2.314†	1.364	1.428	1.273	1.351	1.319	1.385	1.26	1.33	1.303	1.371
H-5α	1.091	1.154					1.902	1.959†	1.395†	1.450	1.396	1.447†	1.25	1.320†	1.087	1.151
H-6α	1.27	1.267†	2.010	2.026	5.352	5.375	5.272	5.243	1.77	1.775†	1.363	1.363†	1.323	1.320†	1.33	1.32
H-6β	1.25	1.238†	2.191	2.196					1.75	1.727†	1.505	1.48	1.194†	1.180	1.30	1.28
H-7α	0.862	0.868	0.864	0.871	1.529	1.536	5.479	5.480	5.158	5.146	1.96*	1.96*	1.745	1.751	1.240	1.25
H-7β	1.654	1.648	1.730	1.723	1.973	1.968					1.97*	1.97*	2.373	2.366	1.887	1.882
H-8β	1.332	1.327	1.423	1.423	1.453	1.446	1.941	1.932†							2.003	1.998
H-9α	0.618	0.636	0.731	0.750	0.927	0.946	1.015	1.030	1.643†	1.659			1.632†	1.651†	0.673	0.691
H-11α	1.481	1.476	1.450	1.449	1.505†	1.497†	1.476	1.472	1.572†	1.568	2.11	2.10	1.587	1.588	1.569	1.562
H-11β	1.279	1.274	1.335	1.337	1.458†	1.455†	1.271	1.265	1.461	1.457	2.06	2.06	1.485	1.477	1.339	1.334
H-12α	1.105	1.115	1.105	1.118	1.157	1.164	1.144	1.156	1.214	1.221	1.375	1.386	1.113	1.117	1.227	1.237
H-12β	1.961	1.959	1.985	1.986	2.012	2.010	1.999	1.997	2.025	2.023	1.978	1.972	1.938	1.936	1.999	1.996
H-14α	0.973†	0.982†	0.954	0.961	0.988	0.996†	1.18	1.187	1.796†	1.803†	2.048	2.053†				
H-15α	1.550	1.550	1.568	1.567	1.574	1.575	1.671	1.670	1.517†	1.523	1.577	1.577	2.183	2.181	5.147	5.150
H-15β	1.033†	1.029†	1.061	1.059	1.069	1.064†	1.19	1.19	1.394†	1.387	1.269†	1.266†	2.246	2.240		
H-16α	1.805	1.808	1.815	1.817	1.829	1.831	1.854	1.854	1.880	1.883	1.885	1.887	1.817	1.817	2.279	2.282
H-16β	1.229	1.227	1.243	1.242	1.260	1.257	1.280	1.278	1.266	1.264	1.312†	1.309†	1.369	1.366	1.896	1.895
H-17α	1.069	1.074	1.068	1.075	1.088	1.093	1.105	1.110	1.200	1.204	1.130	1.136	1.119	1.117	1.498	1.505
H-18	0.647	0.646	0.677	0.676	0.678	0.677	0.691	0.689	0.534	0.533	0.609	0.606	0.840	0.838	0.899	0.898
H-19	0.802	0.816	1.048	1.061	1.008	1.019	0.780	0.794	0.796	0.810	0.950	0.963	0.689	0.704	0.822	0.836
H-20	1.355	1.353	1.363	1.363	1.374	1.373	1.379	1.376	1.361†	1.357†	1.380	1.378†	1.455	1.453	1.577†	1.575†
H-21	0.897	0.897	0.900	0.901	0.914	0.914	0.906	0.906	0.920	0.919	0.923	0.923	0.929	0.929	0.910	0.910
H-22R	1.33	1.33	1.33	1.33	1.34	1.34	1.34†	1.34	1.34	1.35	1.34	1.34	1.384†	1.383†	1.36	1.36
H-22S	0.979	0.981	0.981	0.984	0.993	0.995†	0.985	0.986	0.996†	0.998	0.980	0.982†	1.064	1.063	1.036	1.038
H-23R	1.33	1.33	1.33	1.33	1.33	1.34	1.33	1.34	1.34	1.34	1.34	1.34	1.326†	1.325†	1.36	1.36
H-23S	1.13	1.13	1.14	1.14	1.13	1.14	1.13	1.13†	1.14	1.15	1.14	1.14	1.15	1.16	1.17	1.17
H-24	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.11	1.10	1.10	1.10	1.11	1.11	1.10	1.11	1.11
H-24	1.13	1.13	1.13	1.13	1.14	1.13	1.13	1.14	1.13	1.14	1.14	1.14	1.14	1.14	1.15	1.15
H-25	1.513	1.514	1.513	1.514	1.517	1.517	1.516	1.516	1.519	1.519	1.518	1.519	1.518	1.518	1.522	1.522
H-26	0.859	0.859	0.860	0.860	0.862	0.862	0.861	0.861	0.863	0.863	0.863	0.863	0.863	0.863	0.866	0.865
H-27	0.864	0.864	0.864	0.864	0.867	0.867	0.865	0.865	0.868	0.868	0.867	0.868	0.866	0.866	0.868	0.868
Acetate		2.019		2.049		2.032		2.033		2.027		2.022		2.022		2.022

[a] Data obtained at 500.1 MHz in $CDCl_3$ solution (5 to 100 mM) and referenced to the $Si(CH_3)_4$ signal. Chemical shifts given to two (three) decimal places are generally accurate to ±0.01 (±0.001) ppm except that values marked by † are accurate to about ±0.02 (±0.003) ppm. Stereochemical assignments marked by * are uncertain. R and S denote pro-R and pro-S hydrogens. No stereochemical assignments are given for the C-24 hydrogens.

dow ca. δ_C 15–55 (just encompassing the upfield ^{13}C methylene and methine signals); Gaussian apodization (LB -2; GB 0.4) in t_2 and 90° shifted sine bell apodization in t_1; Fourier transform with 2k points in t_2 and 256 points in t_1, followed by phase correction. Similar parameters were used for HSQC spectra to distinguish olefinic signals except for f_2 (δ_H -0.2 to 7.4), f_1 (just large enough to encompass the olefinic ^{13}C resonances), and the number of t_1 increments (10–20).

Nuclear Overhauser enhancement (NOE) difference spectroscopy was done on non-degassed samples without sample spinning: low-power irradiation for 1.0 s, 90° read pulse, 2.7-s acquisition time, 16 scans per cycle. These conditions were also used to isolate overlapped

Table 2. ^1H NMR chemical shifts of diene free sterols **9a-15a** and acetates **9b-15b**[a]

	$\Delta^{4,6}$		$\Delta^{5,7}$		$\Delta^{5,8}$		$\Delta^{5,8(14)}$		$\Delta^{5,24}$		$\Delta^{6,8}$		$\Delta^{6,8(14)}$	
	9a	**9b**	**10a**	**10b**	**11a**	**11b**	**12a**	**12b**	**13a**	**13b**	**14a**	**14b**	**15a**	**15b**
H-1α	1.295	1.342	1.304	1.361	1.361	1.405	1.218	1.279	1.078	1.132	1.341	1.380	1.137	1.182
H-1β	1.656†	1.685	1.889†	1.897†	1.874†	1.888†	1.770	1.782	1.845†	1.858†	1.682	1.700	1.690	1.708
H-2α	2.058	2.086	1.885†	1.918†	1.886†	1.906†	1.876	1.898	1.838†	1.857†	1.864	1.887	1.874	1.879
H-2β	1.636†	1.737†	1.497	1.569	1.560	1.638	1.467	1.554	1.500	1.585	1.458	1.530	1.481	1.566
H-3α	4.275	5.363	3.639	4.703	3.549	4.614†	3.564	4.641	3.525	4.603	3.665	4.744	3.679	4.767
H-4α	5.353	5.281	2.470	2.500	2.347	2.38†	2.387	2.416	2.294	2.32	1.834	1.873	1.791	1.826
H-4β			2.285	2.362	2.309	2.38†	2.195	2.272	2.235	2.31	1.551	1.619	1.380	1.445
H-5α											2.152	2.199	2.060	2.123
H-6α	5.905	5.900	5.575	5.567	5.435	5.456	5.317	5.329	5.352	5.375	5.377	5.343	5.261	5.231
H-7α	5.642	5.659	5.389	5.384	2.513	2.515†	2.592	2.598	1.529	1.535†	5.817	5.814	6.132	6.133
H-7β					2.562	2.548†	2.555	2.548	1.973	1.969				
H-8β	2.063†	2.062							1.454	1.449†				
H-9α	1.000	1.014	1.969	1.989			1.976	1.999†	0.929	0.948			1.918	1.934
H-11α	1.463	1.464	1.586	1.583	2.111†	2.11	1.617	1.618	1.506†	1.499†	2.220†	2.212†	1.610	1.610
H-11β	1.334	1.334	1.721	1.709	2.166†	2.156†	1.581	1.572	1.460†	1.454†	2.023†	2.011†	1.466	1.459
H-12α	1.142	1.154	1.239	1.247	1.439	1.445	1.192	1.198	1.158	1.167	1.435	1.445	1.265	1.271
H-12β	2.017	2.018	2.092	2.089	2.002	2.000	1.996	1.994	2.011	2.009	2.033†	2.032†	2.011	2.010
H-14α	1.137	1.142	1.880	1.887	2.104†	2.105†			0.989†	0.996†	2.130†	2.136†		
H-15α	1.735	1.736	1.689	1.692	1.620	1.620	2.070	2.069	1.577	1.577	1.704	1.706†	2.388	2.388
H-15β	1.216	1.213	1.393	1.386	1.289†	1.281†	2.212	2.205	1.072†	1.067†	1.322†	1.316†	2.297	2.293
H-16α	1.865	1.867	1.920	1.923	1.915	1.917	1.841	1.841	1.839	1.840	1.945	1.948	1.894	1.894
H-16β	1.299	1.298	1.314	1.313	1.335†	1.331†	1.398	1.397	1.266	1.264	1.365†	1.364†	1.437	1.435
H-17α	1.107	1.113	1.213	1.217	1.168	1.171	1.148	1.150	1.102	1.107	1.175	1.179	1.191	1.192
H-18	0.725	0.724	0.621	0.618	0.652	0.650	0.877	0.875	0.680	0.678	0.576	0.573	0.893	0.892
H-19	0.997	1.010	0.947	0.953	1.189	1.200	0.889	0.899	1.009	1.019	0.793	0.806	0.643	0.657
H-20	1.387	1.386	1.390†	1.387†	1.398	1.396	1.475†	1.473	1.396†	1.394†	1.402	1.400†	1.472†	1.469†
H-21	0.909	0.910	0.941	0.941	0.934	0.934	0.941	0.941	0.937	0.936	0.939	0.939	0.942	0.941
H-22R	1.34	1.34	1.36	1.36	1.35	1.35	1.391†	1.393†	1.408†	1.405†	1.35	1.36	1.386	1.385
H-22S	0.988†	0.989	1.015	1.015	0.992	0.993	1.073	1.074	1.041	1.043†	0.997	0.999	1.065	1.065
H-23R	1.34	1.33	1.35	1.35	1.35	1.35	1.335†	1.335†	1.849	1.847	1.35	1.35	1.330	1.330
H-23S	1.13†	1.14	1.15	1.15	1.15	1.15	1.16	1.16	2.021	2.021	1.16	1.15	1.16	1.16
H-24	1.10	1.10	1.11	1.11	1.11	1.11	1.11	1.11	5.090	5.090	1.10	1.11	1.11	1.11
H-24	1.13	1.13	1.14	1.14	1.14	1.14	1.14	1.14			1.14	1.14	1.14	1.14
H-25	1.516	1.517	1.523	1.523	1.521	1.521	1.523	1.524			1.522	1.522	1.520	1.520
H-26	0.862	0.862	0.867	0.867	0.865	0.865	0.866	0.867	1.682	1.682	0.866	0.866	0.864	0.864
H-27	0.866	0.866	0.871	0.871	0.870	0.870	0.870	0.870	1.602	1.602	0.871	0.870	0.868	0.867
Acetate		2.063		2.043		2.033		2.038		2.031		2.036		2.038

[a] See footnote for Table 1.

multiplets by saturation difference spectroscopy (18, 19). Homodecoupling experiments were carried out non-spinning with the carrier at the decoupler frequency (20) and a relaxation delay of 1.5 s. ^{1}H chemical shifts were determined from 1D spectra (±0.001 ppm), f_2 cross sections of HSQC spectra (±0.003 ppm for isolated asymmetrical multiples) or f_1 positions in COSY-DEC spectra (±0.001 ppm observed precision despite substantially coarser digital resolution). Variation of sterol concentration between 0.1 mM and 100 mM gen-

Table 3. ^{1}H NMR chemical shifts of diene and triene free sterols **16a-23a** and acetates **16b-23b**[a]

	$\Delta^{7,9(11)}$		$\Delta^{7,14}$		$\Delta^{7,24}$		$\Delta^{8,14}$		$\Delta^{8,24}$		$\Delta^{5,7,9\,(11)}$		$\Delta^{5,7,10\,(19)}$		$\Delta^{6,8,14}$	
	16a	**16b**	**17a**	**17b**	**18a**	**18b**	**19a**	**19b**	**20a**	**20b**	**21a**	**21b**	**22a**	**22b**	**23a**	**23b**
H-1α	1.343	1.385	1.067	1.121	1.083	1.131	1.258	1.295	1.215	1.249	1.519	1.567	2.180	2.200	1.401	1.441
H-1β	1.953	1.970	1.847†	1.864†	1.825†	1.837†	1.850†	1.869†	1.756	1.773	1.716†	1.716	2.399	2.396	1.722	1.740
H-2α	1.887†	1.907	1.812†	1.833†	1.797†	1.814†	1.876†	1.895†	1.843†	1.853	1.932	1.952	1.924	1.958	1.896	1.920
H-2β	1.487	1.570	1.408	1.493	1.389	1.475	1.478	1.559	1.441	1.525	1.707†	1.780	1.676	1.731	1.495	1.563
H-3α	3.595	4.681	3.597	4.689	3.596	4.694	3.631	4.707	3.617	4.700	3.607	4.662	3.949	4.941	3.682	4.752
H-4α	1.729	1.765	1.720	1.745	1.718	1.740	1.677	1.720	1.646	1.684	2.377	2.415	2.575	2.571	1.868	1.907
H-4β	1.303	1.372	1.251	1.328	1.272	1.351	1.366	1.428	1.320	1.384	2.468	2.533	2.286	2.365	1.573	1.640
H-5α	1.432	1.483	1.422	1.477	1.398	1.450	1.490	1.545†	1.397†	1.45					2.244	2.294
H-6α	1.89*	1.89*	1.859†	1.858†	1.77	1.77	1.442	1.442	1.367	1.363	5.677	5.694	6.237	6.214	5.482	5.451
H-6β	1.90*	1.88*	1.801†	1.783†	1.75	1.73	1.564	1.546†	1.506	1.47						
H-7α	5.367	5.353	5.750	5.739	5.159	5.145	2.356†	2.356	1.96*	1.96	5.405	5.400	6.034	6.030	6.111	6.111
H-7β							2.098	2.093	1.97*	1.96						
H-9α			1.732†	1.746†	1.645†	1.659†							1.685†	1.676†		
H-9β													2.821	2.808†		
H-11α	5.456	5.449	1.601	1.597	1.574	1.570	2.22	2.21	2.11	2.10	5.513	5.507	1.662	1.656†	2.266†	2.260†
H-11β			1.488	1.481	1.463	1.457	2.18	2.17	2.07	2.06			1.530	1.529	2.167†	2.164†
H-12α	2.107	2.115	1.310	1.318	1.217	1.224	1.397	1.407	1.378	1.388	2.158	2.160	1.298	1.292	1.443	1.455
H-12β	2.290	2.286	2.034	2.032	2.025	2.022	2.024	2.021	1.977	1.974	2.372	2.370	2.003	2.003	2.072	2.069
H-14α	2.162	2.170			1.798†	1.804†			2.046†	2.054†	2.214	2.220†	1.980	1.980		
H-15α	1.752	1.752	5.499	5.505	1.521	1.524	5.360	5.366	1.580	1.580	1.769	1.774†	1.48†	1.47*	5.487	5.492
H-15β	1.389†	1.382			1.397	1.388			1.270†	1.265†	1.419	1.413	1.48†	1.49*		
H-16α	1.949	1.952	2.310	2.314	1.888	1.890	2.353	2.356	1.893	1.895	1.973	1.976	1.875	1.872†	2.392	2.395
H-16β	1.319†	1.317	1.916	1.915	1.274	1.269	2.057	2.055	1.316†	1.314†	1.352	1.350	1.27	1.26	2.086	2.085
H-17α	1.248	1.253	1.583†	1.587	1.216	1.219	1.514	1.523	1.146	1.150	1.270	1.273	1.28	1.27	1.554	1.561
H-18	0.505	0.503	0.829	0.827	0.536	0.533	0.818	0.816	0.610	0.607	0.566	0.563	0.543	0.540	0.817	0.815
H-19	0.902	0.916	0.788	0.801	0.797	0.810	0.991	1.004	0.951	0.963	1.246	1.256	*b*	*b*	0.854	0.867
H-20	1.387	1.384†	1.585†	1.587	1.383	1.380	1.614	1.612	1.404†	1.401†	1.407	1.405†	1.366†	1.365†	1.630	1.628
H-21	0.915	0.915	0.925	0.924	0.942	0.941	0.938	0.938	0.946	0.945	0.922	0.922	0.919	0.919	0.955	0.956
H-22R	1.36	1.36	1.36	1.36	1.417	1.416	1.37	1.37	1.411†	1.411†	1.37	1.37	1.35	1.34†	1.39	1.38
H-22S	1.002	1.004	1.039†	1.041	1.047	1.049	1.049	1.052	1.033	1.034	1.011	1.013†	1.006†	1.002†	1.057	1.059
H-23R	1.35	1.35	1.36	1.36	1.853	1.853	1.36	1.36	1.851	1.850	1.35	1.35	1.34	1.34	1.37	1.37
H-23S	1.15	1.15	1.17	1.17	2.025	2.025	1.17	1.18	2.025	2.025	1.16	1.15	1.14	1.14	1.19	1.18
H-24	1.11	1.11	1.12	1.12	5.092	5.091	1.12	1.12	5.093	5.093	1.11	1.11	1.11	1.11	1.12	1.12
H-24	1.14	1.14	1.16	1.15			1.16	1.16			1.14	1.14	1.14	1.14	1.16	1.16
H-25	1.522	1.523	1.526	1.526			1.527	1.528			1.523	1.524	1.519	1.520	1.531	1.530
H-26	0.865	0.866	0.868	0.867	1.683	1.682	0.868	0.868	1.683	1.682	0.867	0.867	0.864	0.864	0.871	0.870
H-27	0.870	0.870	0.871	0.870	1.602	1.602	0.871	0.870	1.602	1.602	0.872	0.872	0.869	0.869	0.873	0.873
Acetate		2.030		2.028		2.025		2.031		2.025		2.035		2.040		2.042

[a] See footnote for Table 1. [b] Values for H-19: **22a**, 4.822 (pro-Z), 5.050 (pro-E); **22b**, 4.838 (pro-Z), 5.059 (pro-E)

erally had little effect (≤0.001 ppm) on chemical shifts or resolution, but some positions (notably 1β) showed slightly larger variation in chemical shift (0.002 ppm) between 1D spectra (mainly ≤20 mM) and 2D spectra (usually 100 mM). ^1H-^1H coupling constants were derived from line spacings of resolution-enhanced 1D spectra or (at lower precision) from f_2 cross sections of HSQC spectra. Relaxation times measured on non-degassed samples of **3a, 11b,** and **20a** using the inversion-recovery method indicated T_1 values of ≤1 s for all protons except H-3α, H-25, H-26, H-27, and olefinic protons (all ≤2 s). PC Model (Serena Software; Bloomington, IN) was used for modeling of sterols by molecular mechanics and to predict vicinal ^1H NMR coupling constants.

The following sterols were prepared as described previously: **1a** (21), **2a** (22), **3a** (21), **4a** (23), **5a** (24), **6a** (25), **7b** (26), **8b** (27), **16a** and **16b** (28), **17a** and **17b** (29), **18b** (30), **19a** and **19b** (31), **20a** and **20b** (30), **21b** (32), and **23a** (33). The following compounds were purchased: 5α-cholestane from Aldrich; **9a** (93:7 mixture of **9a** and **2a**) from Steraloids, Inc. (Wilton, NH); **10a** (~95% purity) from Chemical Dynamics (South Plainfield, NJ); **13a** (~85% purity) from Sigma Chemical Co. (St. Louis, MO); and **22a** from Sigma. Acetate derivatives were prepared from free sterols by acetylation with pyridine and acetic anhydride overnight at room temperature, and free sterols were prepared from acetates by saponification. Analytical samples were obtained by

Table 4. ^{13}C NMR chemical shifts of monoene free sterols **1a-8a** and acetates **1b-8b**[a]

	Δ⁰		Δ⁴		D⁵		D⁶		D⁷		D⁸		D⁸(¹⁴)		D¹⁴	
	1a	**1b**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**	**7a**	**7b**	**8a**	**8b**
C-1	36.97	36.72	35.33	34.95	37.22	36.96	34.57	34.32	37.11	36.80	35.11	34.86	36.48	36.23	36.94	36.70
C-2	31.49	27.44	29.49	25.07	31.62	27.74	31.39	27.29	31.42	27.47	31.63	27.58	31.54	27.55	31.35	27.36
C-3	71.36	73.76	67.96	70.95	71.77	73.96	71.54	73.70	71.05	73.46	71.21	73.58	71.24	73.68	71.26	73.67
C-4	38.17	33.99	123.20	118.84	42.26	38.09	36.04	31.99	37.92	33.78	38.31	34.15	38.24	34.09	38.05	33.91
C-5	44.82	44.61	147.76	149.66	140.72	139.61	44.96	44.74	40.21	40.02	40.73	40.54	44.25	44.05	44.25	44.06
C-6	28.71	28.58	32.21	32.24	121.70	122.63	130.42	129.98	29.62	29.51	25.47	25.36	28.86	28.74	28.38	28.26
C-7	32.06	31.96	33.08	32.92	31.88	31.87	129.26	129.42	117.39	117.25	27.18	27.09	29.60	29.51	30.02	29.95
C-8	35.47	35.44	35.91	35.86	31.86	31.82	38.11	38.05	139.60	139.54	128.26	128.38	126.28	126.10	34.96	34.94
C-9	54.32	54.18	54.42	54.20	50.08	49.98	52.55	52.39	49.41	49.23	134.95	134.66	49.26	49.16	53.56	53.43
C-10	35.43	35.42	37.30	37.28	36.47	36.56	34.19	34.16	34.17	34.17	35.68	35.66	36.76	36.74	35.58	35.59
C-11	21.23	21.17	20.99	20.94	21.05	21.00	21.10	21.02	21.52	21.46	22.80	22.72	19.95	19.89	21.87	21.83
C-12	40.01	39.94	39.82	39.77	39.74	39.69	40.03	39.96	39.53	39.47	36.91	36.85	37.25	37.21	42.35	42.30
C-13	42.56	42.55	42.43	42.42	42.28	42.28	43.45	43.43	43.35	43.33	42.07	42.06	42.68	42.69	47.05	47.05
C-14	56.46	56.38	56.14*	56.05	56.73	56.65	54.42	54.31	55.01	54.96	51.88	51.82	142.67	142.82	155.57	155.45
C-15	24.20	24.18	24.19	24.18	24.27	24.26	23.93	23.92	22.93	22.92	23.76	23.75	25.79	25.80	117.00	117.14
C-16	28.23	28.22	28.18	28.17	28.22	28.21	28.34	28.33	27.93	27.93	28.76	28.76	27.02	27.02	35.55	35.57
C-17	56.25	56.22	56.12*	56.11	56.10	56.09	56.08	56.05	56.11	56.10	54.84	54.83	56.86	56.86	58.71	58.69
C-18	12.05	12.04	11.94	11.95	11.84	11.83	12.11	12.10	11.83	11.84	11.19	11.21	18.19	18.21	16.82	16.84
C-19	12.30	12.19	18.89	18.79	19.38	19.29	11.36	11.25	13.03	12.93	17.82	17.69	12.79	12.70	12.01	11.91
C-20	35.77	35.78	35.75	35.75	35.77	35.77	35.78	35.78	36.18	36.20	36.26	36.27	34.42	34.43	33.86	33.88
C-21	18.64	18.64	18.62	18.63	18.69	18.69	18.63	18.62	18.82	18.82	18.72	18.72	19.06	19.06	18.91	18.92
C-22	36.14	36.13	36.12	36.11	36.16	36.15	36.15	36.14	36.10	36.09	36.12	36.12	35.95	35.95	36.05	36.05
C-23	23.81	23.81	23.80	23.80	23.80	23.80	23.85	23.84	23.88	23.90	23.91	23.92	23.68	23.69	23.68	23.67
C-24	39.49	39.48	39.47	39.48	39.49	39.49	39.47	39.47	39.47	39.47	39.48	39.48	39.53	39.53	39.51	39.53
C-25	27.99	27.99	27.98	27.99	27.99	28.00	27.98	27.98	27.99	27.99	27.98	27.99	28.00	28.01	28.00	28.02
C-26	22.54	22.54	22.54	22.54	22.55	22.55	22.54	22.54	22.54	22.54	22.53	22.54	22.56	22.56	22.55	22.56
C-27	22.80	22.80	22.80	22.81	22.81	22.81	22.80	22.80	22.81	22.81	22.82	22.81	22.79	22.80	22.79	22.80
Acetate		21.47		21.46		21.44		21.45		21.47		21.47		21.47		21.48
		170.69		171.00		170.53		170.65		170.69		170.69		170.69		170.71

[a] Chemical shifts referenced to the $CDCl_3$ signal at 77.0 ppm. Data obtained at 125.8 MHz in $CDCl_3$ solution (5-150 mM). Asterisks denote assignments that may be interchanged.

argentation chromatography, HPLC, or chromatography on silica gel.

The purity of sterol samples was determined by HPLC, GC, TLC, and/or ^{1}H NMR. Unless otherwise indicated, product ratios and purities are based on ^{1}H NMR data. In the methyl region, levels of minor impurities were estimated by comparison of signal heights with those of ^{13}C satellites of the major component.[2] In the δ 2–6 region, impurity levels were determined by integration. ^{1}H NMR analysis showed a purity of ≥99% for **1a, 1b, 2a, 3a, 4a, 4b, 5a, 6a, 6b, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11a, 11b, 12b, 13a, 13b, 14a, 14b, 15a, 15b, 18a, 19b, 20a, 20b, 21a, 21b, 22a, 23a,** and **23b,** a purity of ≥98% for **2b, 3b, 5b, 7a, 12a, 16b, 17a,** and **22b,** and a purity of ≥97% for **17b** and **18b.** Dienes **16a** and **19a** showed a single component on HPLC and GC. ^{1}H NMR chemical shifts for free sterols **1a–23a** and acetates **1b–23b** (**Fig. 1**) are given in **Table 1,** Table 2, and **Table 3.** The corresponding ^{13}C NMR chemical shifts are given in **Table 4, Table 5,** and **Table 6.** ^{1}H NMR coupling constants are listed in **Table 7, Table 8,** and **Table 9.**

[2]Relaxation times and broadening from unresolved long-range couplings appear to be comparable for H-18 and H-19 bonded to either ^{12}C or ^{13}C as judged by the similarity of the H-18 and H-19 peak heights (usually varying by ≤10%) and the approximately 0.55% intensity of their ^{13}C satellites. When the H-18 and H-19 resonances are isolated from other signals and the line shape at their base is optimal, integration affords better accuracy than peak height comparisons. Under the more common circumstances of imperfect line shapes and proximity of minor signals to the base of a large peak, integration offers no advantage over peak height measurements. Peak height comparisons were not made to the H-21, H-26, or H-27 doublets, which have somewhat narrower lines than those of H-18 and H-19.

Table 5. ^{13}C NMR chemical shifts of diene free sterols **9a-15a** and acetates **9b-15b**[a]

	$\Delta^{4,6}$		$\Delta^{5,7}$		$\Delta^{5,8}$		$\Delta^{5,8\,(14)}$		$\Delta^{5,24}$		$\Delta^{6,8}$		$\Delta^{6,8\,(14)}$	
	9a	**9b**	**10a**	**10b**	**11a**	**11b**	**12a**	**12b**	**13a**	**13b**	**14a**	**14b**	**15a**	**15b**
C-1	33.56	33.23	38.35	37.90	35.60	35.38	36.28	35.97*	37.22	36.96	33.57	33.32	35.12	34.89
C-2	29.09	24.63	31.95	28.08	31.91	27.98	31.78	27.92	31.63	27.75	31.55	27.48	31.44	27.39
C-3	68.08	70.89	70.43	72.79	71.41	73.61	71.15	73.50	71.78	73.97	71.18	73.42	71.43	73.62
C-4	125.94	121.44	40.75	36.62	42.18	38.05	41.78	37.64	42.27	38.09	36.06	32.00	36.55	32.49
C-5	144.99	146.64	139.73	138.49	138.86	137.74	140.86	139.77	140.72	139.62	41.95	41.74	44.73	44.49
C-6	127.91	127.67	119.57	120.19	119.54	120.42	120.60	121.45	121.70	122.63	128.69	128.21	129.37	128.86
C-7	131.73	132.17	116.23	116.24	28.98	28.97	29.26	29.27	31.88	31.87	125.63	125.74	125.72	125.84
C-8	37.37	37.31	141.43	141.60	126.41	126.42	123.33	123.20	31.86	31.83	128.74	128.80	125.20	125.03
C-9	51.36	51.17	46.20	46.00	132.03	131.70	46.54	46.44	50.08	49.99	139.03	138.81	48.12	47.99
C-10	35.10	35.00	36.98	37.05	37.33	37.41	37.43	37.52	36.47	36.57	35.77	35.75	35.66	35.61
C-11	20.70	20.62	21.09	21.00	22.26	22.19	19.35	19.30	21.06	21.00	23.17	23.12	19.71	19.63
C-12	39.80	39.73	39.16	39.10	36.78	36.75	37.66	37.62	39.74	39.69	36.61	36.52	36.74	36.67
C-13	43.39	43.37	42.89	42.87	41.95	41.94	42.75	42.77	42.31	42.31	42.02	41.99	43.63	43.63
C-14	54.02	53.92	54.46	54.43	51.79	51.74	143.34	143.47	56.72	56.64	50.12	50.05	147.53	147.73
C-15	23.78	23.76	23.00	23.00	22.97	22.97	25.98	25.99	24.28	24.27	23.62	23.60	24.92	24.92
C-16	28.21	28.20	28.07	28.08	28.80	28.80	27.18	27.18	28.20	28.19	28.79	28.79	27.34	27.33
C-17	56.06	56.02	55.84	55.83	54.72	54.72	57.21	57.21	56.03	56.02	54.79	54.73	55.88	55.86
C-18	11.96	11.95	11.80	11.81	11.27	11.29	18.56	18.58	11.84	11.84	10.73	10.74	19.23	19.22
C-19	18.05	17.80	16.26	16.15	22.84	22.70	19.04	19.00	19.38	19.30	12.30	12.21	11.30	11.20
C-20	35.77	35.77	36.12	36.14	36.22	36.23	34.44	34.45	35.59	35.60	36.24	36.26	34.76	34.76
C-21	18.63	18.62	18.82	18.82	18.69	18.70	19.04	19.05	18.61	18.61	18.74	18.73	18.87	18.86
C-22	36.13	36.11	36.09	36.09	36.09	36.09	35.98	35.99*	36.07	36.06	36.14	36.12	35.87	35.86
C-23	23.86	23.84	23.85	23.86	23.89	23.90	23.69	23.71	24.69	24.69	23.92	23.91	23.66	23.66
C-24	39.46	39.46	39.47	39.48	39.47	39.47	39.53	39.54	125.19	125.20	39.49	39.48	39.50	39.50
C-25	27.98	27.98	27.99	28.00	27.99	27.99	28.01	28.02	130.93	130.93	27.98	27.99	28.00	28.00
C-26	22.54	22.54	22.54	22.55	22.54	22.53	22.56	22.57	25.73	25.72	22.53	22.54	22.56	22.55
C-27	22.81	22.81	22.81	22.81	22.81	22.81	22.80	22.81	17.63	17.63	22.80	22.82	22.79	22.78
Acetate		21.44		21.42		21.40		21.45		21.44		21.44		21.44
		170.99		170.54		170.45		170.57		170.56		170.68		170.64

[a] See footnote for Table 4.

HPLC purification of desmosterol (**13a;** 85% purity) in SS-7 gave, in addition to **13a** of ≥99% purity, two minor components, which might be anticipated from a reported (34) synthesis of **13a.** Cholesta-5,25-dien-3β-ol: single component by HPLC in SS-7 (t_R 21.5 min; t_R 19.6 min for **13a**), TLC in SS-6 (R_f 0.38; essentially identical to that of **13a**), GC (t_R 42.8 min; t_R 42.8 min for **13a;** 60-m column), and ^{1}H NMR; MS, 384 (76, M^+), 369 (26), 366 (58), 351 (28), 299 (25), 273 (21), 271 (45), 255 (27), 245 (18), 213 (38), 145 (77), 107 (85), 105 (100); ^{1}H NMR δ 5.352 (m, H-6), 4.684 (br s, H-26), 4.661 (br s, H-26), 3.52 (m, H-3α), 2.29 (br d, H-4α), 2.23 (br t, H-4β), 1.711 (br s, H-27), 1.009 (s, H-19), 0.928 (d, 6.5 Hz, H-21), 0.681 (s, H-18). Cholesta-5,22(*E*),24-trien-3β-ol: single component by HPLC in SS-7 (t_R 15.4 min), TLC in SS-6 (R_f 0.38), and GC (t_R 46.6 min; 60-m column); MS, 382 (5, M^+), 364 (4), 300 (7), 285 (5), 282 (11), 271 (27), 255 (12), 253 (9), 213 (5), 109 (100); ^{1}H NMR (~95% purity) δ 6.133 (dd, 15.1, 10.6 Hz, H-23), 5.751 (br d, 10.8 Hz, H-24), 5.402 (dd, 15.0, 8.9 Hz, H-22), 5.349 (m, H-6), 3.52 (m, H-3α), 1.747 and 1.732 (two br s, H-26 and H-27), 1.045 (d, 6.6 Hz, H-21), 1.012 (s, H-19), 0.709 (s, H-18). Purification of crude desmosterol acetate (**13b;** 85% purity) by argentation chromatography gave, in addition to **13b** of ≥99% purity, 3β-acetoxycholesta-5,22(*E*),24-triene: single component by GC (t_R 59.8 min; t_R 55.3 min for **13b;** 60-m column); ^{1}H NMR similar to that of the free sterol except for δ 5.373 (m, H-6), 4.601 (m, H-3α), 2.32 (m, H-4α and H-4β), 2.031 (s, Ac-CH_3), 1.021 (s, H-19), 0.706 (s, H-18). The $\Delta^{5,25}$ and $\Delta^{5,22E,24}$ NMR data are compatible with those reported previously for the acetate derivatives (35).

*Cholesta-5,8-dien-3β-ol (**11a**), cholesta-5,8(14)-dien-3β-ol (**12a**), and their acetates (**11b** and **12b**).* In a modification

Table 6. ^{13}C NMR chemical shifts of diene and triene free sterols **16a-23a** and acetates **16b-23b**[a]

	$\Delta^{7,9(11)}$		$\Delta^{7,14}$		$\Delta^{7,24}$		$\Delta^{8,14}$		$\Delta^{8,24}$		$\Delta^{5,7,9(11)}$		$\Delta^{5,7,10(19)}$		$\Delta^{6,8,14}$	
	16a	**16b**	**17a**	**17b**	**18a**	**18b**	**19a**	**19b**	**20a**	**20b**	**21a**	**21b**	**22a**	**22b**	**23a**	**23b**
C-1	34.65	34.36	36.78	36.50	37.11	36.81	35.30	35.04	35.11	34.87	38.27	38.01	31.91	32.17	33.98	33.76
C-2	31.60	27.58	31.46	27.54	31.46	27.47	31.68	27.63	31.66	27.58	32.21	28.27	35.13	31.96	31.45	27.43
C-3	70.93	73.32	70.92	73.33	71.06	73.46	70.98	73.32	71.22	73.58	72.20	74.02	69.17	71.78	71.06	73.22
C-4	37.73	33.61	37.83	33.85	37.97	33.78	38.28	34.11	38.33	34.15	41.54	37.39	45.83	42.12	36.04	32.01
C-5	39.19	38.97	39.65	39.48	40.22	40.02	40.92	40.73	40.74	40.55	141.23	139.96	134.99	134.28	41.60	41.43
C-6	29.87	29.73	30.25	30.14	29.62	29.51	25.28	25.14	25.48	25.36	118.27	119.06	122.44	122.43	129.90	129.41
C-7	120.23	120.02	120.15	119.95	117.43	117.28	26.58	26.49	27.19	27.09	115.56	115.53	117.44	117.44	124.23	124.33
C-8	136.29	136.28	134.48	134.41	139.58	139.51	123.05	123.13	128.26	128.37	135.48	135.65	142.34	142.48	123.25	123.29
C-9	143.85	143.54	49.72	49.55	49.41	49.23	140.84	140.47	134.96	134.68	144.11	143.93	29.01	29.05	142.52	142.22
C-10	35.67	35.65	33.84	33.74	34.18	34.17	36.52	36.48	35.69	35.67	39.27	39.28	145.04	144.61	36.56	36.54
C-11	118.54	118.66	20.95	20.89	21.53	21.45	21.84	21.76	22.80	22.73	122.56	122.59	23.57	23.56	22.08	22.01
C-12	42.29	42.26	40.12	40.06	39.53	39.47	36.93	36.85	36.91	36.85	43.01	42.98	40.51	40.52	36.12	36.06*
C-13	42.31	42.30	46.44	46.42	43.39	43.36	45.02	45.00	42.10	42.09	42.23	42.22	45.89	45.90	44.90	44.88
C-14	51.54	51.48	152.02	151.90	55.01	54.95	151.07	150.98	51.88	51.82	50.91	50.87	56.32	56.35	148.35	148.27
C-15	23.17	23.15	119.42	119.58	22.95	22.94	117.42	117.58	23.78	23.76	22.80	22.80	22.24	22.21	118.19	118.32
C-16	28.39	28.39	35.13	35.14	27.91	27.90	35.90	35.89	28.74	28.74	28.48	28.47	27.65	27.66	35.96	35.96
C-17	56.31	56.30	58.67	58.66	56.03	56.01	57.21	57.17	54.74	54.74	56.34	56.33	56.55	56.56	57.16	57.14
C-18	11.21	11.24	16.47	16.49	11.84	11.85	15.67	15.68	11.20	11.22	11.35	11.36	11.98	11.96	15.53	15.54
C-19	19.44	19.34	12.34	12.24	13.04	12.93	18.35	18.22	17.83	17.70	30.43	30.21	112.42	112.71	13.33	13.23
C-20	35.95	35.97	34.09	34.10	35.99	36.00	34.03	34.03	36.07	36.07	35.91	35.91	36.13	36.12	34.09	34.08
C-21	18.39	18.39	18.89	18.90	18.74	18.74	18.86	18.85	18.64	18.64	18.37	18.37	18.83	18.83	18.92	18.92
C-22	36.04	36.04	36.04	36.04	36.00	36.00	36.08	36.07	36.03	36.02	36.02	36.01	36.11	36.13	36.08	36.08*
C-23	23.90	23.91	23.73	23.74	24.75	24.76	23.73	23.71	24.78	24.78	23.87	23.87	23.85	23.85	23.77	23.76
C-24	39.47	39.47	39.47	39.48	125.17	125.17	39.49	39.49	125.19	125.19	39.46	39.44	39.48	39.48	39.48	39.48
C-25	27.99	27.99	27.98	27.99	130.97	130.96	27.99	27.99	130.93	130.91	27.99	27.98	28.01	28.01	28.00	27.99
C-26	22.54	22.54	22.53	22.54	25.73	25.72	22.55	22.54	25.72	25.72	22.54	22.53	22.55	22.55	22.55	22.54
C-27	22.81	22.82	22.79	22.79	17.64	17.64	22.79	22.79	17.63	17.62	22.82	22.80	22.81	22.81	22.80	22.79
Acetate		21.45		21.44		21.47		21.44		21.47		21.37		21.42		21.40
		170.66		170.64		170.69		170.66		170.69		170.41		170.64		170.59

[a] See footnote for Table 4.

Table 7. ^{1}H-^{1}H NMR coupling constants for monoene free sterols **1a-8a** and acetates **1b-8b**[a]

	Δ^0		Δ^4		Δ^5		Δ^6		Δ^7		Δ^8		$\Delta^{8(14)}$		Δ^{14}	
	1a	**1b**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**	**7a**	**7b**	**8a**	**8b**
1α-1β	13.3	13.3	13.3	13.4	13.2†		13.2		12.9†		13.1	13.2	13.2	13.3		13.7†
1α-2α	4.0†	3.5†	2.8	2.8	3.8†		4.3†		4.0‡		4.2	4.0†	3.8†	4.0†	4.3†	3.9†
1α-2β	13.8	14.0†	14.0†	14.2†	13.6†		13.5†		14.1‡		13.9†	14.0†	14.0†	14.0†		13.8†
1β-2α	3.3†	3.5†	4.7	4.8	3.1†♦	♦	2.7†		3.3‡		3.6†	3.4†	3.5†	3.3†	3.3†	3.4†◊
1β-2β	4.0	4.1	2.8	3.0	3.1†		4.4	◊	3.3‡		3.6†	3.9†	3.8†	4.0†	3.9	3.7†
2α-2β	12.7		12.3	12.3†			12.9†								12.6†	
2α-3α	4.8†	4.9†	6.0	6.0†	4.2		5.1†	5.2†	4.5†	4.6†	4.9†	5.0†	4.8†	4.9†	4.7†	4.9
2β-3α	11.3	11.5†	9.8	10.3†	11.2		11.0	11.1†	11.2†	11.1†	10.8†	11.2†	11.0†	11.3†	11.4	11.4†
3α-4α	5.0†	4.9	2.0	2.5‡◊	5.2		4.8	4.9	4.8†	4.6†	4.8†	5.0†	4.8‡	4.9‡	4.7†	4.9
3α-4β	11.5†	11.2			11.1		10.8	11.1	11.0	11.2	10.8†	11.1†	11.0‡	11.3‡	11.0	11.2
4α-4β	12.4†	12.3			13.0◊	♦	12.3	12.2	12.4†	12.4†	11.8†	11.6‡			12.5†	12.4
4α-5α	2.5†	3.0					2.9	2.8	3.5‡		2.2‡	2.1‡				3.1
4β-5α	12.4†	12.3†					13.4	13.6	12.4†	12.4†	◊	11.7‡◊	♦	♦	12.5†	12.6†
5α-6α							2.1†	2.1†						♦		
5α-6β	13.4‡												◊			
6α-6β	13.4‡♦	♦	13.8	13.8					♦	♦					♦	◊
6α-7α		5.9†	4.2	4.3	2.0†		9.9	9.9	5.0*							
6α-7β	3.0†	2.8	2.4	2.5	5.1	5.2†										
6β-7α	13.4‡	12.3‡	13.9	13.9					2.3†*				2.1†	2.0†	◊	♦
6β-7β	3.9†	3.8†	4.9	4.9									4.4†	4.7†		
7α-7β	13.0†	12.9	12.6		17.2	17.2†					♦	♦	14.1†	14.1†		
7α-8β	11.3‡	11.0‡	11.5‡	11.5‡	10.8†	11.5‡	2.5†	2.4†								
7β-8β	4.0†	3.8†	3.7	3.8	4.9	5.1†										
8β-9α	10.5	10.5	10.6	10.6	10.9	10.7	10.4†	10.0							11.7†	11.7†
8β-14α	10.8†	11.0‡	10.8	10.6	10.3	10.4†										
9α-11α	4.2	4.1	4.2	4.2	5.8†	5.2†	3.7†	3.6					◊	7.0	3.0†	3.1†
9α-11β	12.5	12.4	12.4	12.4	12.0‡	10.7†	12.8†	12.5					10.3†		11.7†	11.7†
11α-11β	13.5	13.5‡	13.6†	13.6	13†◊	◊	13.0	13.2	13.5‡		♦	♦	13.4†	13.4	13.2†	13.2
11α-12α	4.3	3.9†	4.4	4.4†	4.8†	4.5†	4.3†	4.1†	4.5‡				3.6†	3.5†	3.7†	3.8†
11α-12β	2.9	3.0	3.1	3.0	3.3†	3.5‡	3.0†	3.0†	2.9				3.3†	3.5†	3.2†	3.2†
11β-12α	13.6†	13.8†	13.5†	13.5†	12.7†	12.7‡	13.5†	13.4†	13.4†				13.8†	13.8†	13.1‡	
11β-12β	4.0	3.9	4.1	4.1	4.0	3.5‡	3.7	3.6	4.3			7.3†	3.6†	3.5†	3.2†	3.2†
12α-12β	12.6	12.6	12.9	12.5	12.8	12.5†	12.7	12.5	12.7			12.4†	12.5	12.5	12.6	12.6
14α-15α	6.6†		7.1	7.1	6.8	6.8†										
14α-15β	13.0†◊	◊	12.7	12.7	12.7	12.6†◊	♦	♦	12.1‡	12.1‡						
15α-15β				12.7‡	12.5‡	11.8	12.1‡			12.1‡	12.1‡		17.0◊	17.0◊		
15α-16α			9.2†	9.5†	9.9	9.7†			9.3†				9.1	9.1	3.2	3.1
15α-16β	3.1		3.0†	3.1	3.1	3.2				2.8			8.9†	8.9†	1.7*	1.7*
15β-16α				6.0	6.0	6.2	6.0			6.2†	6.6†		2.6	2.5		
15β-16β				12.7‡	11.4	11.3	11.1			12.1‡	11.8†◊	◊	10.8	10.8		
16α-16β	13.0†		13.2	13.3	13.2	13.3			12.6†	13.2			12.8	12.8	15.5	15.4
16α-17α	9.5†	9.9‡	9.8†	9.5†	9.4†	9.7†	9.8‡	9.8‡	9.3‡	9.5‡	9.6‡	9.6‡	7.1	7.1	7.6	7.6
16β-17α	9.9‡	9.9‡	9.9‡	9.6	9.5	9.6	9.8‡	9.8‡	9.6‡	9.0	9.6‡	9.6‡	12.4†	12.4†	10.4	10.3
17α–20	9.9‡	9.9‡	9.9‡	10.3†	10.4†	10.3†	9.8‡	9.8‡	9.6‡	9.5‡	9.6‡	9.6‡	9.6†	9.6†	10.5†	10.4†

[a] ^{1}H-^{1}H spin coupling constants (absolute values) obtained at 500 MHz in $CDCl_3$ solution at 25°C. Accuracy is estimated to be ±0.2 Hz except for couplings marked by † (±0.5 Hz) or ‡ (ca. ±1 Hz). Strongly coupled pairs of protons are indicated by ♦ ($\Delta\nu/J$ <1.5) or ◊ (1.5 ≤$\Delta\ \nu/J$ <3). Other couplings observed for essentially all sterols: $J_{2\alpha\text{-}4\alpha}$, ~2.2 Hz; $J_{19\text{-}1\alpha}$, ~0.6 Hz (often not resolved); $H_{18\text{-}12\alpha}$, ~0.5 Hz (often not resolved); $J_{20\text{-}21}$ ~6.6 Hz; and smaller unresolved couplings for $J_{18\text{-}12\beta}$, $J_{18\text{-}17\alpha}$, $J_{19\text{-}1\beta}$, $J_{21\text{-}17\alpha}$, and $J_{21\text{-}22R}$. Sterols with a C_8H_{17} side chain showed, $J_{24R\text{-}25}$, 6.6† Hz; $J_{24S\text{-}25}$, 6.6† Hz; $J_{25\text{-}26}$, 6.6 Hz; $J_{25\text{-}27}$, 6.6 Hz; and Δ^{24} sterols showed $J_{23R\text{-}24}$, 7.1† Hz; $J_{23S\text{-}24}$, 7.1† Hz; $J_{24\text{-}26}$, 1.4 Hz; $J_{24\text{-}27}$, 1.4 Hz; and unresolved couplings for $J_{23R\text{-}27}$, $J_{23S\text{-}27}$, and $J_{26\text{-}27}$.

[b] Additional couplings (all in Hz; uncertain assignments are marked by an asterisk): **2a** and **2b**: 2α-4, 1.6; 3α-6β, 1.9; 4-6β, 1.9; **3a**: 4β-6, 2.1; 4β-7α, 3.0†; 4β-7β, 2.6; **4a** and **4b**: 5α-7, 2.5†; 6-8β, 2.1†; **5a** and **5b**, 7-9α, 2.3‡; 7-14α, 2.3‡; **7a** and **7b**: 7α-15α, 2.5‡, 7α*-15β, 2.1‡; 9α-15α, 2.5‡, 9α*-15β,2.1‡; **8a** and **8b**: 8β-16α, 1.8†; 8β-16β, 4.0.

of a procedure reported for preparing **11a** (36), outlined in **Fig. 2, 10b** was treated with diethyl azodicarboxylate, and the resulting crude mixture of ene adducts (7.43 g; **24** and **25** containing unreacted diethyl azodicarboxylate) was separated by MPLC on silica gel (Lobar column; elution with ethyl acetate–hexane 1:9 (2900 ml), ethyl acetate–hexane 12:88 (4200 ml), and ethyl acetate–hexane 1:3. Fractions 267–332 contained the $\Delta^{5,8(14)}$ adduct **25** (0.90 g), fractions 333–358 contained a mixture of **24** and **25** (0.84 g), and fractions 359–392 contained the $\Delta^{5,8}$ adduct **24** (2.21 g).

Reduction of **25** (0.50 g) as described previously for **24** (36) gave a crude product that was filtered through silica gel (elution with ethyl acetate–hexane 12:88); fractions 7–15 contained crude **12b** (298 mg), which was acetylated and subjected to MPLC on alumina-$AgNO_3$ (1 m × 10 mm i.d. column; elution with toluene–hexane 1:9). Evaporation of fractions 99–165 gave **12b** (99 mg). Recrystallization from methanol furnished an analytical sample of **12b**: mp 76.5–77.5°C; single component on TLC in SS-2 (R_f 0.55, identical to that of **11b**) and SS-4 (R_f 0.73, identical to that of **11b**); IR, υ_{max} 3000–2800, 1728, 1466, 1371, 1265, 1242, 1049, 1028 cm^{-1}; MS, 426.3520 (3, M^+; calcd. for $C_{29}H_{46}O_2$, 426.3498), 411 (2, M–CH_3), 366.3319 (100, M–CH_3COOH), 351.3075 (19, M–CH_3COOH–CH_3), 313.2150 (5, M–SC), 281 (2), 253.1953 (18, M–CH_3COOH–SC), 227 (4), 211.1478 (7), 199.1487 (8), 158 (13), 145 (7), 143 (7), 131 (4), 107 (3), 105 (6); >99% purity by ^{1}H NMR (contains 0.2% **11b**); ^{1}H and ^{13}C NMR, Tables 2, 5, and 8. A sample of crude **12a** from another reaction was subjected to argentation chromatography to give **12a** of 98% purity: mp 131–132.5°C; >99% purity by GC (t_R 20.5 min; 30-m column); IR, υ_{max} 3400, 3000–2800, 1464, 1368, 1061, 1024 cm^{-1}; MS, 384.3374 (100, M^+; calcd. for $C_{27}H_{44}O$, 384.3392), 369.3232 (19, M–CH_3), 366 (15, M–H_2O), 351.3051 (77, M–H_2O–CH_3), 325 (11), 271.2073 (39, M–SC), 253.1942 (29, M–H_2O–SC), 227 (13), 217.1549 (11), 211.1485 (27), 199.1455 (23), 159.1157 (25), 158.1088 (22), 157.1018 (28), 145.1009 (36), 143.0854 (34), 105.0702 (39); ^{1}H and ^{13}C NMR, Tables 2, 5, and 8.

Similarly, reduction of **24** followed by acetylation and chromatographic purification gave **11b:** mp 102.2–102.8°C (lit. mp 100–101°C (36)); single component by TLC in SS-6, HPLC (t_R 28.1 min; elution with methanol), and GC (t_R 27.0 min; 30-m column); ≥99% purity by ^{1}H NMR. Reduction of another sample of **24** followed by chromatographic purification gave **11a:** single component by TLC in SS-6 and GC (t_R 20.8 min; 30-m column); ~99% purity by ^{1}H NMR.

*3β-Benzoyloxy-5α-cholesta-6,8-diene (**14c**).* In an extension of reactions described previously (31) and outlined in **Fig. 3,** hydrogen chloride gas was bubbled through a solution of **10c** (6.61 g) in toluene (300 ml) at -70°C for 30 s. The mixture was held at -70°C for an additional 90 s, and poured into cold concentrated aqueous ammonia. Pyridine (1 ml) was added, and the aqueous layer was removed. After addition of more pyridine (1 ml), the toluene layer was evaporated to an oily yellow solid. This material was dissolved in chloroform (20 ml containing a trace of pyridine), acetone (60 ml) was added, and crystals slowly began forming. The mixture was placed in a -20°C freezer for 15 min, followed by suction filtration to give shiny white crystals (2.04 g), consisting of a mixture of **26, 17c, 10c, 15c** (~12:4:3:2) and traces of **3c, 14c,** 5β-**15c,** and unidentified components. A second crop (0.86 g) consisted mainly of **26, 17c, 10c,** and **15c,** and the mother liquor (2.38 g, oil) contained 5β-**15c, 10c, 15c, 17c, 26** (3:1:1:1:1 ratio), and small amounts of unidentified sterols.

A portion (1.01 g) of the first crop of crystals was dissolved in chloroform (50 ml), sodium carbonate (5 g) was added, and the mixture was stirred vigorously at room temperature for 5 days. NMR analysis showed a mixture of **14c, 10c, 15c, 3c,** and unidentified sterols (41:24:19:6:10 ratio) and complete disappearance of **26.** The reaction mixture was filtered, and pyridine (2 drops) was added to the filtrate, which was evaporated to a white solid (926 mg). The mixture was adsorbed onto silica gel (4 g) and subjected to MPLC on silica gel-$AgNO_3$ (1 m × 10 mm i.d. column; elution with toluene–hexane 1:9 (3500 ml) and toluene). Fractions 14–19 contained **3c** (20 mg). Subsequent fractions contained mixtures of **14c, 15c,** and 5β-**15c:** fractions 47–50 (35 mg; 95:0:5); fractions 51–60 (24 mg; 94:2:4); fractions 61–65 (24 mg; 89:7:4); fractions 66–83 (16 mg; 89:9:2); fractions 84–123 (40 mg; 2:1:0); fractions 124–169 (41 mg; 1:1:0). The toluene eluate contained **10c, 17c, 15c,** and **14c** in a 7:7:4:2 ratio. The latter three fraction sets contained a few percent of an unidentified sterol (^{1}H NMR, δ 5.74 (br d, 7 Hz), 5.54 (br d, 7 Hz),

Table 8. ^{1}H-^{1}H NMR coupling constants for diene free sterols **9a-15a** and acetates **9b-15b**[a]

	$\Delta^{4,6}$		$\Delta^{5,7}$		$\Delta^{5,8}$		$\Delta^{5,8\,(14)}$		$\Delta^{5,24}$		$\Delta^{6,8}$		$\Delta^{6,8\,(14)}$	
	9a	**9b**	**10a**	**10b**	**11a**	**11b**	**12a**	**12b**	**13a**	**13b**	**14a**	**14b**	**15a**	**15b**
1α-1β		13.2			13.4†	13.7‡	13.5	13.5	13.5†		13.2†	13.1	13.1	13.2
1α-2α					4.2†		3.6†	3.8	4.0†		3.7†	3.5†	3.9†	3.6
1α-2β	13.0‡	13.0‡			13.9‡	12.9‡	14.0	14.1†	14.0†		14.0†	13.9†	14.0†	13.7
1β-2α	3.6†	3.5†	♦	◊	3.4†◊	3.7†◊	3.5†	3.6†	3.8†♦	♦	3.4†	3.3†	3.0†	3.5†
1β-2β	3.1†	3.4†			3.4†	3.8†	3.5	3.6†	3.8†		3.9	4.0†	4.2	4.4
2α-2β	13.0‡	13.0‡				12.9‡	12.5	12.4			~13†			12.9
2α-3α	7.0‡	7.0‡		4.0	5.4		4.1†	4.3			4.9†	5.0†	5.0	5.1†
2β-3α	9.0‡	9.5‡		11.4†	11.0	11.6†	11.1	11.5†			11.2	11.3‡	10.9†	11.6
3α-4α	2.4‡	2.7	4.8	5.0	5.3		4.9	5.0	5.1		4.8	5.0†	4.9	4.9
3α-4β			11.7	11.5†	10.9		11.3†	11.7	11.2		10.9	11.2	10.9	11.1
4α-4β			14.3	14.5	12.6♦	♦	13.8	13.8	13.1◊	♦	12.5	12.5	12.3	12.3
4α-5α											3.4	3.5†	3.2	3.2
4β-5α											13.8	13.9	13.5	13.7
5α-6α											2.6	2.2	2.2	3.1
6α-7α	9.9	9.9	5.7	5.7	4.5‡		3.7†		2.0†		9.4	9.4	9.8	9.8
6α-7β					3.0‡		3.7†	3.8†	5.2					
7α-7β					22.5♦	22.5†♦	21.5†♦	21.2†♦	17.2					
7α-8β	2.2	2.1							◊	11.3‡				
7β-8β									5.0	5.2†				
8β-9α	9.9	10.2†							11.3‡	11.0†				
8β-14α	12.1‡	11.5†							10.6†	10.3				
9α-11α	3.9	4.1†	7.2				7.4	7.4	5.4†	5.0†			5.9	5.9
9α-11β	12.6	12.2†	11.8	11.9			10.2	10.1	12.0‡	11.9†				11.4
11α-11β	13.3	13.2	13.9	13.7†	♦	♦	13.8♦	13.7◊	13.2‡◊	◊			13.3	13.4†
11α-12α	3.6†	3.9†	4.5	4.7	8.0‡	8.0‡	4.9	4.6	4.9	4.8			3.2	3.3
11α-12β	3.0	3.1	2.6	2.7	2.0	1.9	3.4	3.3	3.1†	3.2			3.8†	3.9
11β-12α	13.2‡	13.3†	13.5†	13.4†		11.0‡	13.0	13.7	12.9†	12.7†			13.7†	13.5†
11β-12β	3.9	3.9	4.9	4.9	7.1	7.1	3.7	3.6	4.0†	4.0	♦	◊	3.2†	3.0
12α-12β	12.8	12.8	12.8	12.8	12.7	12.6	12.5	12.5	12.7	12.7			12.6	12.7
14α-15α	6.9†	6.9†	6.4	7.1		7.1†			7.0†	6.6				
14α-15β	12.1‡	12.8†◊	11.6†	11.9‡	11.9‡	11.5‡			13.2‡	12.5◊				
15α-15β	11.2†	11.5‡	11.5†	11.9‡	11.9‡	11.5‡	17.1	17.0	12.2‡	12.3‡			17.4	17.4
15α-16α	9.7†	9.4†	9.2	9.6†			9.3	9.3	9.7†	9.8			9.8	9.7
15α-16β	2.7†		2.0†	2.8†		2.5†	9.1†	8.9†	3.1	3.0			7.6	7.6
15β-16α	5.6	5.6	6.1	6.0†		6.4†	2.1	2.0	6.1†	6.1			3.2	3.1
15β-16β		11.5‡	11.6†	11.3†◊	11.9‡◊	11.5‡◊			11.0‡	11.1	◊	◊	11.3	11.3†
16α-16β	12.9	12.8	12.7	12.3†			12.8	12.8	13.3	13.4			13.0	13.0
16α-17α	9.3†	9.4†	9.8	9.6†	9.7‡	9.7‡	7.1	7.0	9.7†	9.1	9.1‡	9.4‡	7.6	7.6
16β-17α	10.2†	9.7‡	9.7†	9.2	9.7‡	9.7‡	12.4†	11.8	9.5	9.5	9.1‡	9.4‡	11.5	11.4
17α-20	10.2†	9.7‡	9.7†	9.6†	9.7‡	9.7‡	9.7†	9.6	9.9‡	9.8‡	9.1‡	9.4‡	9.7	9.6

[a] See footnote *a* for Table 7.

[b] Additional couplings (all in Hz; uncertain assignments are marked by an asterisk): **9a** and **9b**: 4-2α, 1.5†; 4-6, 0.7; 4-7*, 0.7; 4-8β*, 0.7; 6-8β, 2.8; **10a** and **10b**: 4β-6, 2.7; 4β-9α, 2.7‡; 4β-14α, 2.7‡; 4β-7, ≤0.5; 7-9α, 2.8†; 7-14α, 0.6†; **11a** and **11b**: 4β-6, 1.8†; 4β-7α, 3.2; 4β-7β*, 1.8†; 7α-11α*, ~2‡; 7α-11β, ~2‡; 7α-14α*, ~2‡; 7β-11β*, ~2.5‡; 7β-14α*; ~1†; **12a** and **12b**: 4β-6, 2.1; 4β-7α, 3.7; 4β-7β, 3.7†; 7α-15α, 2.8‡; 7α-15β*, 1.8‡; 7α-9α, 1.8‡; 7β-15α, 1.4; 7β-15β*, 1.4†; 7β-9α, 1.4‡; 9α*-15α, 1.4‡; **13a** and **13b**: 4β-6, 2.0; 4β-7α, 3.0‡; 4β-7β, 2.7; 23R-24, 7.1†; 23S-24, 7.1†; 23R-26, 1.3; 23S-26, 1.3; 24-26, 1.3; 24-27, 1.1; **14a** and **14b**: 5α-7, 3.2; **15a** and **15b**: 5α-7, 1.0†; 7-15α, 0.7†; 7-15β*, 3.1; 9α*-15α, 2.4; 9α*-15β, 0.7†.

Table 9. ^{1}H-^{1}H NMR coupling constants for diene and triene free sterols **16a-23a** and acetates **16b-23b**[a]

	$\Delta^{7,9(11)}$		$\Delta^{7,14}$		$\Delta^{7,24}$		$\Delta^{8,14}$		$\Delta^{8,24}$		$\Delta^{5,7,9(11)}$		$\Delta^{5,7,10(19)}$		$\Delta^{6,8,14}$	
	16a	**16b**	**17a**	**17b**	**18a**	**18b**	**19a**	**19b**	**20a**	**20b**	**21a**	**21b**	**22a**	**22b**	**23a**	**23b**
1α-1β	13.2	13.3	13.2	13.2	13.1†	13.5†	13.0†	13.6‡	13.1	13.2		13.6	13.8	13.7	12.6	12.9
1α-2α	3.7†	3.8†	3.8†			3.7†	4.2†	4.3†	3.9	3.9†		3.9	4.7	4.6	3.6†	3.7†
1α-2β	14.1†	14.1	13.9†			13.9†	14.0†	13.6‡	13.9†	13.9		13.8†	8.5	9.3	13.8†	14.0†
1β-2α	3.5†	3.6†	3.5†	3.5†	3.5†	3.6†	3.6†		3.6†	3.7†		2.9	7.9	7.2	3.3†	3.5†
1β-2β	3.7†	3.8	3.5†	3.5†	3.5†	3.6†	3.6†		3.6†	3.7†	♦	4.1	4.8	4.7	3.3†	3.5†
2α-2β	12.5†	12.6								12.7			12.7	13.1†	12.1†	
2α-3α	4.6†	4.8†	4.5†	4.8†	4.4†	4.6†	5.0	4.9†		5.0†	4.8†	4.8†	3.4	3.5	4.8†	4.9†
2β-3α	11.2†	11.5	11.2†	11.4†	11.1	11.5†	11.1†	11.3†		11.6	11.0†	11.4†	8.0†	8.2†		11.3†
3α-4α	4.6†	4.8†	4.6	4.8	4.7	4.6†	4.8†	4.8	4.7	4.9	5.0	5.1	3.9	4.2	4.8†	4.9†
3α-4β	11.0	11.2	11.0	11.4	11.0	11.2	11.0	11.3†	4.7	10.8†	11.2	11.4†	7.5	8.2†	11.0	11.3
4α-4β	12.5	12.2†	12.4	12.3	12.3	12.4†	12.1	12.2	11.8	11.8†	12.8	12.9	13.1	13.3	12.4†	12.6
4α-5α			3.6	3.6	3.5		2.4†	2.3†	2.3†						3.4†	3.6
4β-5α	12.5	12.7	12.6†	12.6†	12.5	12.4†	12.2†	12.2‡	12.4†◊	12.0†◊					13.7	13.9
5α-6α									◊						2.5	2.6
5α-6β							12.0‡◊	♦		♦						
6α-6β	♦	♦	18.7†◊	◊	♦	♦	12.0‡									
6α-7α			5.3	5.2	4.9	5.2					6.0	6.0	11.3	11.3	9.5	
6α-7β							2.7	2.0‡								
6β-7α			2.4	2.5†	2.3†	2.2†	12.0‡									9.5
6β-7β							6.4	5.0‡								
7α-7β							16.9	16.8‡	♦	♦						
9α-11α			4.3‡										◊	◊		
9α-11β			12.3		12.4											
11α-11β			13.6†	13.2‡	13.4†	13.6‡	♦	♦	16.5‡♦	♦					19.5†	19.6†
11α-12α	2.0†	2.0†	3.6†	3.6†	4.4†	4.3†	6.8†	6.7†	8.3‡		2.3†	2.3†	4.3†		6.1†	6.1†
11α-12β	6.7	6.7	3.4†	3.5†	2.8		2.2	2.1	2.0‡		6.6	6.6	2.6	2.6	1.7	~1‡
11β-12α			13.5†	13.4†	13.4†	13.6†	11.9†	12.2‡	10.0‡				14.3‡	13.1‡	11.8†	12.0†
11β-12β			3.4	3.5†	4.4	4.0†	5.2	5.1	7.4†	6.9			3.9	4.0	5.7	6.0†
12α-12β	17.3	17.2	12.9	12.7	12.7	12.6	12.9	12.2‡	12.9†	12.2	17.7	17.6	12.9	12.7†	12.8	12.9†
14α-15α	7.9				6.8						7.8†					
14α-15β	11.2	11.7‡			12.0‡	12.0‡			12.0‡	11.9‡	11.6‡	11.6‡				
15α-15β	12.2†	11.7‡			12.3	12.0‡			12.1‡	11.9‡	12.2†	11.6‡	♦	♦		
15α-16α			3.5	3.4	9.6		3.4	3.4			10.0†				3.4	3.4
15α-16β	2.8†		1.9	1.9	2.7		2.3‡	2.3‡			2.7†				2.5†	2.5†
15β-16α	5.7†	5.6†			6.4†	6.7†			6.1	5.9†	5.7					
15β-16β	12.0‡◊	11.7‡◊			12.0‡	12.0‡			12.1‡◊	11.9‡◊	11.6‡◊	11.6‡◊				
16α-16β			15.9	15.9	12.6†		16.4	16.3	12.7						16.3	16.4
16α-17α	9.3‡	9.4‡	7.3†	7.3†	9.6†	9.5‡	7.6	7.3	9.6†	9.4‡	9.5‡	9.5‡			7.4	7.5
16β-17α	9.3‡	9.4‡	9.8†		9.5‡	9.5‡◊	10.8	10.9†	9.0‡	9.4‡	9.5‡	9.5‡	♦	♦	10.4†	10.4†
17α-20	9.3‡	9.4‡	♦	♦	9.5‡	9.5‡	10.6	10.8	9.7‡	9.4‡	9.5‡	9.5‡			10.7	10.4†

[a] See footnote a for Table 7.

[b] Additional couplings (all in Hz; uncertain assignments are marked by an asterisk): **16a** and **16b**: unassigned couplings for H-7, m; H-11, dt, 6.7, 2.0 Hz; H-14α, ddq, 11.2, 7.9, 2.9 Hz; **17a** and **17b**: 7-9α, 2.4†; **18a** and **18b**: as for **5b** (steroid nucleus) or **13b** (side chain); **20a** and **20b**: as for **5b** (side chain); **21α** and **21β**: 4β-6, 2.0; 4β-?, 1.9; 7-11, 2.1†; 7-14α, 1.9‡; 7-12α, 1.9‡; **22a** and **22b**: 4α-6†, 1.4; 4β-6†, 1.4; 7-9α, 1.7†; 7-14α, 1.7†; 19E-19Z, 2.6; 19E-1α, 1.4†; 19E-1β, 1.4†; **23a** and **23b**: 5α-7, 3.2; 7-15*, 0.8.

5.43 (br d, 7 Hz), 4.93 (tt, 10, 4 Hz), 0.579 (s)). Fractions were combined based on HPLC analysis and the following retention times (elution with methanol–isopropyl alcohol 1:1): 5β-**15c** (7.2 min), **17c** (8.6 min), **15c** (9.4 min), **14c** (9.8 min), **10c** (10.6 min). Similar dehydrochlorination of the remaining crude **26** followed by MPLC gave analogous results with an increased yield (160 mg) of **14c** containing mainly 5β-**15c.** This material and that from the first column were dissolved in chloroform (1 ml containing 0.5 μl pyridine) and precipitated by dropwise addition of acetone (1 ml). The crystals were stored briefly at -20°C and collected by filtration (two crops) to afford an analytical sample of **14c** (150 mg): mp 148°C, clearing at 190°C (lit. mp 146°C, clearing at 180°C (37)); single component on TLC in SS-1 (R_f 0.50) and SS-5 (R_f 0.18); MS, 488 (16, M^+), 426 (4), 366 (56, M–C_6H_5COOH), 351 (73, M–C_6H_5COOH–CH_3), 325 (14), 313 (3), 253 (9, M–C_6H_5COOH–SC), 211 (16), 157 (21), 145 (29), 143 (53), 105 (94); ~98% purity by ^1H NMR; ^1H NMR, δ 8.05, 7.55, 7.43, 5.836, 5.380, 5.007, 0.948, 0.874, 0.870, 0.861 (s), 0.589 (non-aromatic multiplicities and couplings as for **14b**); ^{13}C NMR, similar to that of **14b** (±0.05 ppm) except for C-1, δ 33.39; C-2, 27.61; C-3, 74.01; C-4, 32.13; C-5, 41.82; C-8, 129.15; C-10, 35.82; C-19, 12.29; and benzoyl signals, 128.25, 129.52, 130.77, 132.72, 166.08.

*5α-Cholesta-6,8-dien-3β-ol (**14a**).* To a solution of **14c** (113 mg) in degassed ethanol (40 ml) was added potassium hydroxide (2.64 g). The reaction was stirred under nitrogen at room temperature for 3 h, when TLC indicated completion of reaction. The reaction was neutralized with 5% sulfuric acid, followed by extraction with dichloromethane, drying over Na_2SO_4, and evaporation to a white solid (126 mg). The crude product was filtered through silica gel (1 g; elution with hexane, followed by ethyl acetate–hexane 1:4). Evaporation of solvent followed by recrystallization from dichloromethane–methanol gave an analytical sample of **14a** (27 mg): mp 123–123.5°C (lit. mp 120–122°C (37)); single component on TLC in SS-3 (R_f 0.50); >99% purity by ^1H NMR; ^1H and ^{13}C NMR, Tables 2, 5, and 8. Evaporation of the mother liquor gave additional **14a** (30 mg).

*3β-Acetoxy-5α-cholesta-6,8-diene (**14b**).* Reaction of **14a** (30 mg) with acetic anhydride (0.5 ml) in pyridine (0.5 ml), followed by pouring into water, extraction with ethyl acetate, and evaporation of solvent gave a solid (32 mg). Recrystallization from dichloromethane–methanol gave an analytical sample of **14b** (18 mg): mp 109–110°C, clearing at 122°C (lit. mp 111–112°C, clearing at 118–120°C (37)); single component on TLC in SS-3 (R_f 0.87); MS, 426.3477 (38, M^+; calcd. for $C_{29}H_{46}O_2$, 426.3498), 366.3272 (77, M–CH_3COOH), 351.3048 (100, M–CH_3COOH–CH_3), 325.2885 (14), 313.2171 (3, M–SC), 311.2759 (4), 311.1920 (3), 253.1930 (8, M–CH_3COOH–SC), 211.1486 (16), 197.1327 (16), 157.1017 (25), 145.1017 (26), 143.0863 (52); ≥99% purity by ^1H NMR; ^1H and ^{13}C NMR, Tables 2, 5, and 8.

*3β-Acetoxy-5α-cholesta-6,8(14)-diene (**15b**).* Using a procedure described previously for the free sterol (31), a solution of **10b** (4.97 g) in benzene (130 ml) containing 2,5-dihydrothiophene-1,1-dioxide (3.59 g) and pyridine (1.5 ml) was heated in a sealed tube at 110°C for 45 h to give a 4:1 mixture of **15b** and **10b.** After evaporation of solvents and crystallization from methanol, the mixture (3.88 g) was subjected to MPLC on silica gel-$AgNO_3$ (500 mm × 25 mm i.d. column; elution with 1.3% ethyl acetate in hexane). Fractions 27–70 contained **15b** (1.54 g): mp 106.5–107°C (lit. mp 106–108°C (38)); MS, 426.3499 (45, M^+; calcd. for $C_{29}H_{46}O_2$, 426.3498), 411.3267 (26, M–CH_3), 366.3285 (26, M–CH_3COOH), 351.3049 (35, M–CH_3COOH–CH_3), 341.2484 (3), 313.2157 (82, M–SC), 299.2018 (12), 281.2276 (4), 265.1958 (7), 253.1960 (85, M–CH_3COOH–SC), 227.1793 (61), 211.1484 (35), 199.1488 (100), 105 (69); ≥99% purity by ^1H NMR; ^1H and ^{13}C NMR, Tables 2, 5, and 8.

Stability of sterols in $CDCl_3$ solution. A solution of **3a** (1.4 mg) and 5α-cholestane (1.2 mg) in $CDCl_3$ (99.8% D; 0.5 ml; stored refrigerated for 2 months in a screw-cap bottle) was kept in a 5-mm NMR tube in a cabinet at

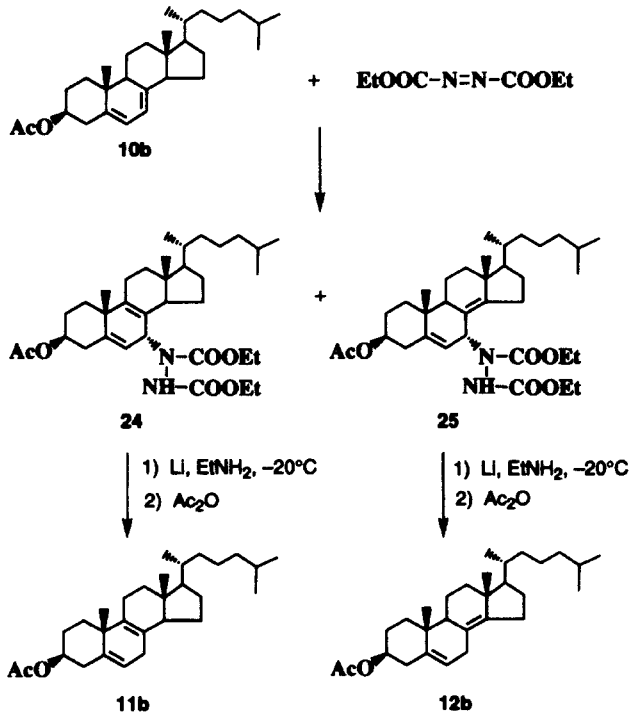

Fig. 2. Chemical synthesis of 3β-acetoxycholesta-5,8-diene (**11b**) and 3β-acetoxycholesta-5,8(14)-diene (**12b**) from 7-dehydrocholesterol acetate (**10b**).

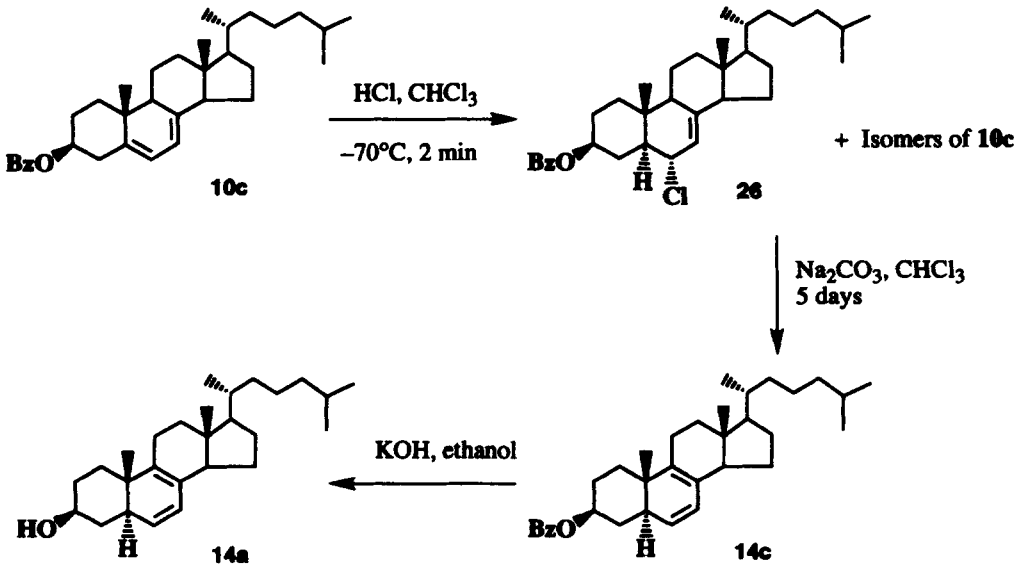

Fig. 3. Chemical synthesis of 5α-cholesta-6,8-dien-3β-ol (**14a**) from 7-dehydrocholesterol benzoate (**10c**).

22°C for 10 days, and ^{1}H NMR spectra were taken after 0.01, 0.5, 1.0, 1.7, 2.7, 3.7, 7.0, and 10.7 days. The ratio of the peak heights of the resolved methyl singlets of **3a** to those of 5α-cholestane was calculated at each time. Under the assumption that 5α-cholestane is stable, the percentage of remaining **3a** was estimated by dividing this ratio by the ratio at the initial time point. Analogous experiments were done for **3b, 7b, 10a, 10b, 14b,** and **15a.** The results are shown in **Fig. 4.** Analogous experiments for **3b** using $CDCl_3$ (99.96% D) from sealed ampules showed: 17 h (99%), 69 h (91%), 185 h (52%) (solvent from Cambridge Isotope Laboratories); 4 h (93%), 30 h (85%), 149 h (66%) (solvent from Aldrich); and 8 h (91%), 29 h (84%) (solvent from Aldrich).

Aliquots (20 μl) from a solution of 5α-cholestane (1.1 mg), **3b** (1.3 mg), **5b** (1.1 mg), and **10b** (1.4 mg) in hexane (19.7 ml) were evaporated to dryness in a stream of nitrogen, subjected to high vacuum for 10 min, and dissolved in $CDCl_3$ immediately prior to NMR analysis. $CDCl_3$ from screw-cap bottles gave spectra with the anticipated signals (**Fig. 5,** panel A), whereas $CDCl_3$ from sealed ampules gave variable results, with spectra often showing complete disappearance of all anticipated methyl signals except those of 5α-cholestane (Fig. 5, panel C). NMR analysis after storage of these samples in $CDCl_3$ solution for 4 days at 22°C indicated no decomposition ($CDCl_3$ from screw-cap bottles) or 80% decomposition of **10b** (solvent from a sealed ampule). No sterol decomposition was noted by NMR after storage of the hexane solution for a week at 22°C. Other samples, stored prior to NMR analysis as a thin sterol film in a 1-dram vial at 22°C in air, showed either no decomposition (sample stored for 7 days) or minor decomposition of **10b** only (sample stored for 3 days; Fig. 5, panel D).

These results were consistent with numerous other experiments in which nanogram or low microgram amounts of unsaturated sterols dissolved in $CDCl_3$ from sealed ampules usually showed none of the expected NMR signals. For example, dichloromethane solutions of **3b** containing 26 μg, 4 μg, and 1.6 μg were evaporated to dryness in 1-dram vials and left in a desiccator containing D_2O, with exposure to air and light over 1 day. ^{1}H NMR analysis was done with $CDCl_3$ from sealed ampules. The 1.6- and 4-μg samples showed no **3b** and mainly an unidentified sterol having signals at δ 4.35 (d, 3.5 Hz), 2.56 (dd, 11, 14 Hz), 2.042 (s), 1.375 (s), 0.908 (d, 6.4 Hz), 0.698 (s), and the 26-μg sample was mainly a ~1:1 mixture of **3b** and the unidentified component. In another experiment, hexane solutions containing **3b** (1 mg; 100 μg; 10 μg) and 5α-cholestane (550 μg; 55 μg; 5.5 μg) were evaporated to dryness in 1-dram vials and stored in air for 15 h under fluorescent lighting. ^{1}H NMR analysis of the samples in $CDCl_3$ from a screw-cap bottle showed unchanged ratios of **3b:** 5α-cholestane. A fourth sample (1 μg of **3b** and 550 ng of 5α-cholestane) stored in air for 30 h showed a ~1:1 mixture of 5α-cholestane and the unidentified sterol and no **3b** by NMR analysis in $CDCl_3$ from a sealed ampule. An analogous experiment with **10b** gave parallel results except that NMR analysis of the 1 μg-sample showed complete disappearance of **10b.** In these experiments with $CDCl_3$ (99.96% D) from sealed ampules, one lot from Aldrich consistently produced complete decomposition of sterols, one lot from Cambridge Isotope Laboratories usually gave considerable decomposition, and one lot (containing excessive moisture) from Cambridge sometimes

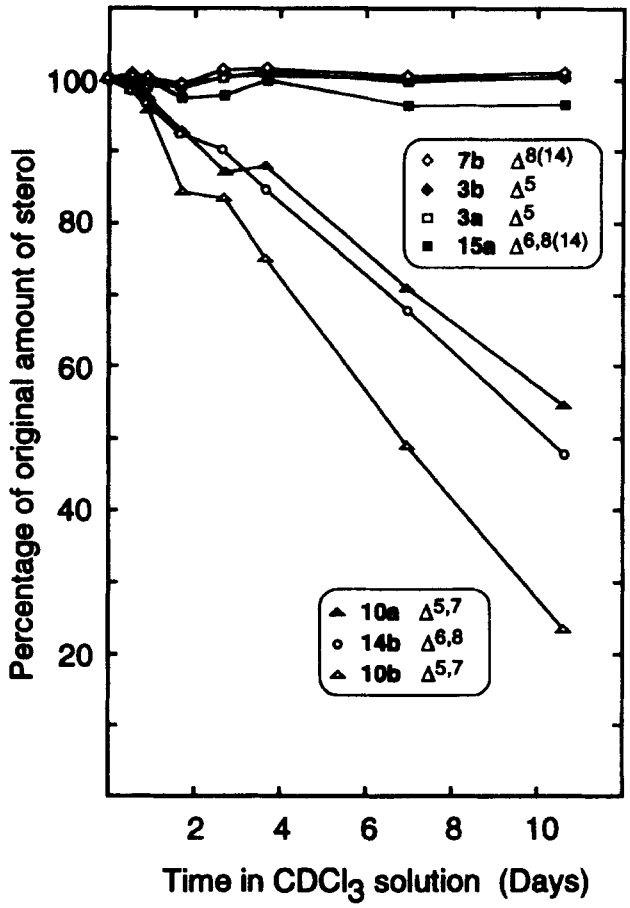

Fig. 4. Stability of sterols **3a, 3b, 7b, 10a, 10b, 14b,** and **15a** at 22°C in $CDCl_3$ solution as measured by ^1H NMR signal intensities relative to those of 5α-cholestane. The symbols for **3b** are almost completely obscured by symbols for **3a** and **7b.** $CDCl_3$ (99.8%) was from screw-cap bottles.

gave decomposition. The $CDCl_3$ (99.8% D) producing no sterol decomposition included two lots stored for several months at 22°C.

RESULTS AND DISCUSSION

Preparation of unsaturated sterols

Chemical syntheses of unsaturated sterols typically give isomeric mixtures that are not easily separated by recrystallization, chromatography on silica gel, or reversed phase HPLC. These mixtures can be separated very effectively by argentation chromatography (12), which was crucial in the isolation of the $\Delta^{6,8}$ benzoate, separation of $\Delta^{5,7}$ and $\Delta^{6,8(14)}$ acetates, partial separation of $\Delta^{7,24}$ and $\Delta^{8,24}$ acetates, and removal of monoene and triene contaminants from preparations of $\Delta^{5,8}$ and $\Delta^{5,8(14)}$ sterols. The $\Delta^{5,8(14)}$ sterol **12a** and its acetate **12b,** which have not been reported previously, were prepared from the known ene adduct **25** (36) under conditions similar to those used in the synthesis of the $\Delta^{5,8}$ sterol (Fig. 2).

The $\Delta^{6,8}$ sterol, which had once been obtained as a minor byproduct in an early synthesis of vitamin D_3 (37), is no longer commercially available. We have previously detected the $\Delta^{6,8}$ benzoate **14c** as the major component in a mixture of sterol dienes obtained by dehydrohalogenation of 3β-benzoyloxy-6α-chloro-5α-cholest-7-ene (**26**), an intermediate in the low-temperature isomerization of 7-dehydrocholesterol benzoate in HCl–chloroform (31). Chloro intermediate **26** is a major component in early stages of the isomerization, but it is gradually converted to dienes, mainly the $\Delta^{7,14}$ and $\Delta^{8,14}$ isomers (31). In the present work, a brief isomerization was carried out in toluene, which appeared to give somewhat lower amounts of the undesired $\Delta^{7,14}$ sterol than chloroform or dichloromethane. The crude product, consisting of **26** and a complex mixture of dienes, was recrystallized to remove most of the 5β sterols, followed by dehydrohalogenation under conditions known to favor formation of the desired $\Delta^{6,8}$ sterol over the $\Delta^{5,7}$ and $\Delta^{6,8(14)}$ isomers (31). The resulting product was freed of isomeric contaminants by MPLC on silica gel-$AgNO_3$. The foregoing reactions, illustrated in Fig. 3, represent a two-step synthesis of the $\Delta^{6,8}$ sterol from 7-dehydrocholesterol benzoate, an inexpensive starting material. The low yield resulted from competing formation of other dienes and from the tedious chromatographic separation of the $\Delta^{6,8}$ and $\Delta^{6,8(14)}$ benzoates.

Assignment of ^1H and ^{13}C NMR chemical shifts

^1H and ^{13}C NMR spectra, DEPT, HSQC, COSYDEC, and, in many cases, NOE difference and HMBC spectra were available for establishing signal assignments of the unsaturated sterols. Together with chemical shift comparisons, these data provided multiple criteria for assigning most signals. Of various possible approaches to the signal assignments (10), the following strategy based mainly on the HSQC spectrum was simple and direct. As illustrated in **Fig. 6,** HSQC spectra consistently gave f_2 cross sections that reproduced the 1D splitting pattern of individual protons without major intensity or phase distortions.[3] After compensation for the much lower

[3]In our hands, f_2 cross sections of HSQC spectra show substantially higher purity of absorptive phase than cross sections of HMQC (heteronuclear multiple quantum coherence) spectra. However, others have obtained good ^1H splitting patterns for a bile acid from a ^{13}C-coupled HMQC spectrum by using a long acquisition time and high digital resolution (39). ^{13}C-decoupled spectra normally require short acquisition times (and correspondingly low digital resolution) to maintain a low decoupler duty cycle and to prevent uneven sample heating, which is indicated by fluctuations in the deuterium lock signal and by degraded resolution. By limiting the f_1 window to about 40 ppm, we could decrease the GARP decoupling power to a level where the lock signal is stable even at a decoupler duty cycle of 33%. ^1H-^1H couplings could frequently be estimated from f_2 cross sections of these spectra, but the accuracy was modest owing to low digital resolution and slightly dispersive line shapes.

resolution in the HSQC spectra, this feature permitted the facile assignment of most protonated carbons based on distinctive ^1H splitting patterns noted by Kirk et al. (40). Resonances showing unusual or nondescript coupling patterns, including many allylic resonances broadened by multiple unresolved long-range couplings, were identified from correlations to assigned protons in the COSYDEC spectrum or by process of elimination. Pairs of protonated olefinic carbons or closely spaced aliphatic carbons were differentiated by HETCOR or by a separate HSQC experiment with a very small f_1 window as described previously (41). Pairs of quaternary olefinic carbons were distinguished by HMBC results or by chemical shift comparisons. Some assignments required combinations of these methods. For example, the olefinic ^1H and ^{13}C assignments of the Δ^6 acetate (**4b**) were established by correlation of the downfield olefinic proton to C-5, C-8, and C-9 in an HMBC experiment and to the upfield olefinic carbon in an HSQC spectrum.

The foregoing strategy generally gave full signal assignments except for stereochemistry of some methylene protons, which was deduced from the splitting patterns, NOE difference experiments, or chemical shift comparisons. For example, NOE effects observed after irradiation of H-18 or H-19 differentiated the broadened H-11α and H-11β signals in sterols containing a Δ^8 double bond and the corresponding H-12 signals of the $\Delta^{7,9(11)}$ sterol. An NOE difference experiment on the $\Delta^{5,8(14)}$ acetate with irradiation of H-15β (3.7 Å from H-7α and 2.5 Å from H-7β) showed a large enhancement for the upfield C-7 proton, demonstrating that this is H-7β. An analogous NOE difference experiment distinguished H-7α from H-7β in the $\Delta^{5,8}$ sterol **11b.** These assignments were compatible with representative acetylation-induced shifts for H-7α (0.006 ppm) and H-7β (-0.006 ppm) of other sterols in Tables 1–3. Stereochemical assignments for the C-22 and C-23 proton signals in sterols with a C_8H_{17} side chain were made by chemical shift comparisons (42).

Stereochemical assignments have not been reported for side-chain protons of Δ^{24} C_{27} sterols. The H-22 signals were assigned by chemical shift comparisons with sterols having a C_8H_{17} side chain (42), and these assignments were consistent with observed couplings to the C-22 protons in the $\Delta^{5,24}$ and $\Delta^{8,24}$ sterols. The C-23 protons were assigned by a series of homodecoupling experiments on zymosterol (**20a**) indicating a 9-Hz coupling to H-22S (δ 1.03) from the upfield C-23 proton (δ 1.85) and a 4.8-Hz coupling from the downfield proton (δ 2.02). These findings were confirmed by a double-quantum-filtered COSY experiment, in which the active couplings in the δ 1.03–1.85 and δ 1.03–2.02 cross peaks corresponded to ~10 Hz and ~5 Hz, respectively. Assuming a predominately extended side-chain conformation, which is indicated by the H-22S coupling constants,[4] these results demonstrate that the resonance at δ 1.85 corresponds to the C-23 pro-R proton and the signal at δ 2.02 corresponds to the pro-S proton. These stereochemical assignments were extended to desmosterol and the $\Delta^{7,24}$ sterol by chemical shift comparisons and should apply to other sterols with the same Δ^{24} side chain, such as lanosterol.

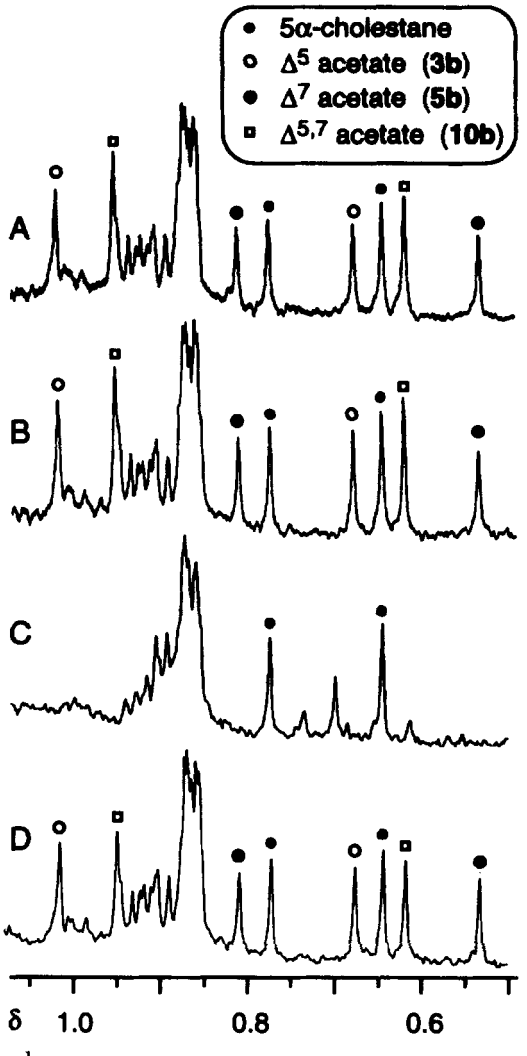

Fig. 5. ^1H NMR spectra of a mixture of 5α-cholestane (1.1 μg), **3b** (1.3 μg), **5b** (1.1 μg), and **10b** (1.4 μg) evaporated from hexane, dissolved in $CDCl_3$, and analyzed by NMR (1-s acquisition time; 75° pulse; 23-min acquisition; exponential multiplication with 1-Hz line broadening): panel A, $CDCl_3$ (99.8% D) from a screw-cap bottle; panels B and C, $CDCl_3$ (99.96% D) from sealed ampules. NMR spectra in panels A–C were acquired shortly after evaporation of hexane. A fourth sample was evaporated from hexane but allowed to stand at 22°C for 4 days prior to NMR analysis in 99.8% D $CDCl_3$ from a screw-cap bottle (panel D). In several additional experiments, $CDCl_3$ from screw-cap bottles gave spectra virtually identical to those of panel A, whereas $CDCl_3$ from sealed ampules gave variable results, of which panels B and C represent the extremes.

^{13}C NMR assignments of lower precision have been reported for many of the sterols described herein. The present ^{13}C chemical shifts are compatible with our recent data for **3a** (42), **10b** (11), and **19b** (31) as well as data from Tsuda and Schroepfer (22) for **2a**, **2b**, **3a**, **3b**, **5a**, **5b**, **6a**, **6b**, **7a**, **7b**, **17a**, **17b**, and **19a** (except for transposition of C-11 and the acetate methyl of **2a**), Eggert and Djerassi (43) for **4a** and 5α-cholest-14-ene (except for interchange of C-6 and C-7 of **4a** and C-16 and C-20 of 5α-cholest-14-ene), Batta et al. (44) for **10a**, **11a**, and **19a** (except for interchange of C-5 and C-8 of **10a** and an error for C-19 of **11a**), Joseph-Nathan, Mejía, and Abramo-Bruno (45) for **13a** (except for a small error for C-15), De Simone et al. (46) for **15a** (except mainly for interchange of C-1, C-4, C-12, and C-22), Taylor et al. (30) for **20a** and **20b** (except for different numbering for C-26 and C-27), Kruk, Jans, and Lugtenburg (47) for **22a,** and Parish, Tsuda, and Schroepfer (33) for **23a.** Full ^{1}H NMR assignments (of lower precision) have apparently been reported only for **3a** (42), **10b** (11, 40), and **19b** (31), although data exist for the methyl and olefinic signals of most of the other sterols.

The ^{13}C NMR shieldings of non-polarizable carbons were reproducible to ca. ±0.01 ppm at the temperature and concentration range specified. Reproducibility was somewhat lower (ca. ±0.04 ppm) for C-3 and olefinic and acetyl carbons. ^{1}H shieldings of sterols appeared to be reproducible to ±0.001 ppm (except for acetate methyls), but generally only first-order resonances identifiable in 1D or COSYDEC spectra could be measured to this precision. Most other ^{1}H signals were determined to ±0.003 ppm from HSQC spectra,[5] but many strongly coupled protons, including almost all the side-chain methylene protons, could be estimated to only ±0.01 ppm. Concentration appeared to have little effect (generally ≤0.001 ppm for ^{1}H) on chemical shifts for $CDCl_3$ solutions of ≤100 mM, as did lowering the temperature from 25°C to 22°C (mainly ≤0.002 ppm for ^{1}H and ≤0.03 ppm for ^{13}C). The high precision of both ^{1}H and ^{13}C data, representing an order of magnitude improvement

[4]Molecular modeling (PCMODEL) indicated significant populations of both *anti* and *+gauche* conformers of the C20-C22-C23-C24 dihedral. (The *-gauche* conformer is unfavorable because of proximity of the C-20 methyl.) Of the ^{1}H NMR couplings to H-22S (13.9, 10.2, 9.2, 4.8 Hz), the 13.9 Hz coupling is geminal. Homodecoupling experiments showed that one of the two large vicinal couplings (10.2 and 9.2 Hz) is to H-20 and therefore that the C17-C20-C22-C23 dihedral is predominantly *anti*. The remaining couplings are to the C-23 protons. The larger one (9.2 or 10.2 Hz) indicates that one C-23 proton is predominantly *anti* to H-22S; this result precludes predominance of a *+gauche* C20-C22-C23-C24 dihedral because H-22S is *gauche* to both C-23 protons in that conformer.

[5]For weakly coupled protons, the precision was nearly ±0.001 ppm, but reliable chemical shifts were difficult to estimate from HSQC cross sections for asymmetrical signals showing second-order effects. If the asymmetry was caused by virtual coupling, chemical shifts were estimated from COSYDEC spectra under the assumption that the signals appear at the true f_1 chemical shift. When a pair of tightly coupled protons showed distinct bands of intensity in COSYDEC spectra (often with an additional band in the center (13)), the bands appeared slightly farther apart than their true chemical shift difference measured from 1D spectra. For example, bands for the C-15 protons of **7a** (Δν/J, 1.9) appeared at δ 2.178 and 2.250 with a center band at δ 2.215, and the C-4 protons of **3a** (Δν/J, 2.2) showed analogous bands at δ 2.233, 2.264, and 2.296.

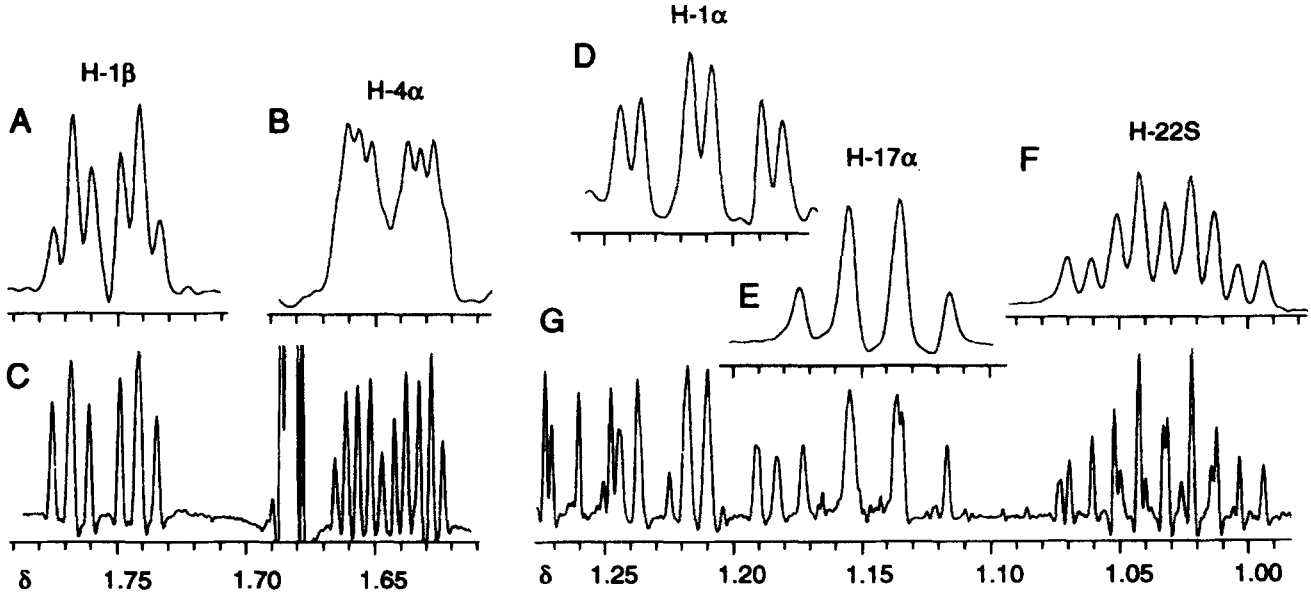

Fig. 6. Comparison of individual resonances from HSQC cross sections (panels A, B, D, E, and F) with signals in the resolution enhanced ^{1}H NMR spectrum of **20a** (panels C and G). All spectra are plotted at the same horizontal scale.

Table 10. Conformation of rings A, B, C, and D of 3β-hydroxysterols having unsaturation in the steroid nucleus[a-c]

Unsaturation	Ring A		Ring B		Ring C		Ring D		Δ	Φm	D_{18-19} (Å)	Twist (°)
	Conformation	μ(Torsion)	Conformation	μ(Torsion)	Conformation	μ(Torsion)	Conformation	μ(Torsion)				
Δ^{0}	C	55.3	C	56.1	C	56.2	13β,14α-HC	30.1	9.3	47.2	4.78	2.1
Δ^{1}	4β,5α-HC/5α-S	31.2	C	56.6	C	55.8	13β,14α-HC	29.9	10.1	47.0	4.72	1.2
Δ^{4}	1α,2β-HCP8	30.8	C	54.1	C	55.7	13β,14α-HC	29.9	9.6	46.9	4.74	3.7
Δ^{5}	C	52.8	8β,9α-HC	30.2	C	54.0	13β,14α-HC	30.1	8.9	47.3	4.69	7.2
Δ^{6}	C	55.1	9α,10β-HC	33.0	C	56.0	13β,14α-HC	30.2	9.2	47.4	4.82	-9.8
Δ^{7}	C	54.8	5α,10β-HC	52.7	C	31.7	13β,14 α-HC	30.1	2.2	47.1	4.53	11.9
Δ^{8}	C	55.8	5α-S	32.3	13β-S	33.5	13β,14α-HC	30.0	2.6	48.4	4.49	2.0
$\Delta^{8(14)}$	C	55.3	C	53.8	12α-S	29.8	13β,17α-HC	24.7	69.8	38.2	4.16	-11.9
$\Delta^{9(11)}$	C	55.5	C	53.0	13β,14α-HC	32.5	13β,14α-HC/13β-E	28.7	16.9	45.2	4.62	-8.2
Δ^{11}	C	55.6	C	56.9	8β,14α-HC/14α-S	34.2	13β,14α-HC	31.2	7.3	49.1	4.52	12.2
Δ^{14}	C	55.3	C	55.8	C	52.1	17α-E	16.1	106.0	26.2	5.00	4.0
Δ^{15}	C	55.3	C	56.2	C	56.3	13β-E	26.7	30.4	42.5	4.72	2.9
Δ^{16}	C	55.3	C	56.2	C	56.1	14α-E	24.4	-27.2	38.5	4.70	1.6
$\Delta^{4,6}$	1α,2β-HC/1α-S	30.3	9α,10β-HC/9α-S	30.0	C	56.6	13β,14α-HC	30.0	9.6	47.1	4.80	-5.8
$\Delta^{5,7}$	C	50.8	9α,10β-HC	15.8	DC	49.0	13β,14α-HC	29.0	3.7	46.9	4.42	16.0
$\Delta^{5,7,9(11)}$	C	54.8	P[d]	3.0	13β-S[d]	29.6	13β,14α-HC	30.1	8.8	47.2	4.53	24.4
$\Delta^{5,8}$	C	52.7	7β,10β-B†	10.8	13β-S	32.8	13β,14 a-HC	31.2	3.7	48.8	4.48	7.7
$\Delta^{5,8(14)}$	DC	50.4	9α-S	26.4	12α-S	29.2	13β,17α-HC	24.3	66.7	37.5	4.15	-12.7
$\Delta^{6,8}$	C	55.7	5α,10β-HC†	25.3	13β-S	32.0	13β,14α-HC	30.4	5.2	47.7	4.41	-6.0
$\Delta^{6,8,14}$	C	55.6	5α,10β-HC†	25.0	12α,13β-HC	29.4	17α-E	17.5	103.3	28.4	4.73	-9.7
$\Delta^{6,8(14)}$	C	55.3	10β-S	31.2	11β,12α-HC/12α-S	32.2	13β-E	24.4	48.6	38.6	4.45	-18.5
$\Delta^{7,14}$	C	54.8	5α,10β-HC	31.9	DC	43.9	17α-E	18.4	101.8	29.7	4.65	1.0
$\Delta^{7,9(11)}$	C	55.3	5α-S	28.4	13β-S	24.9	17α-E	16.0	104.8	27.6	4.64	-3.7
$\Delta^{8,14}$	C	55.6	5α,6β-HC/5α-S	33.0	12α,13β-HC	29.7	17α-E	18.0	102.2	29.1	4.77	3.3

[a] Conformations are based on structures obtained by molecular mechanics using PCMODEL. The conformational type and the average of the absolute values of the five or six internal torsion angles (μ(torsion)) are given for each ring. Chair (C), distorted chair (DC), half-chair (HC), planar (P), boat (B), sofa (S), envelope (E), and intermediate conformational types are distinguished according to criteria in the Atlas of Steroid Structure (ref. 48).

[b] Values of Δ (phase angle of pseudorotation) and ϕ_m (amplitude of puckering) were calculated for ring D according to Altona, Geise, and Romers (ref. 49). D_{18-19} refers to the distance between C-18 and C-19 (a rough measure of the convexity) and the "twist" denotes the C18-C13-C10-C19 pseudotorsion angle.

[c] Distortion is denoted by †; flattening is indicated by torsion angle averages of <54° (chair) or <28° (most other conformations).

[d] A least squares plane defined by all carbon atoms of rings B and C except C-13 showed <0.04 Å deviations for these atoms.

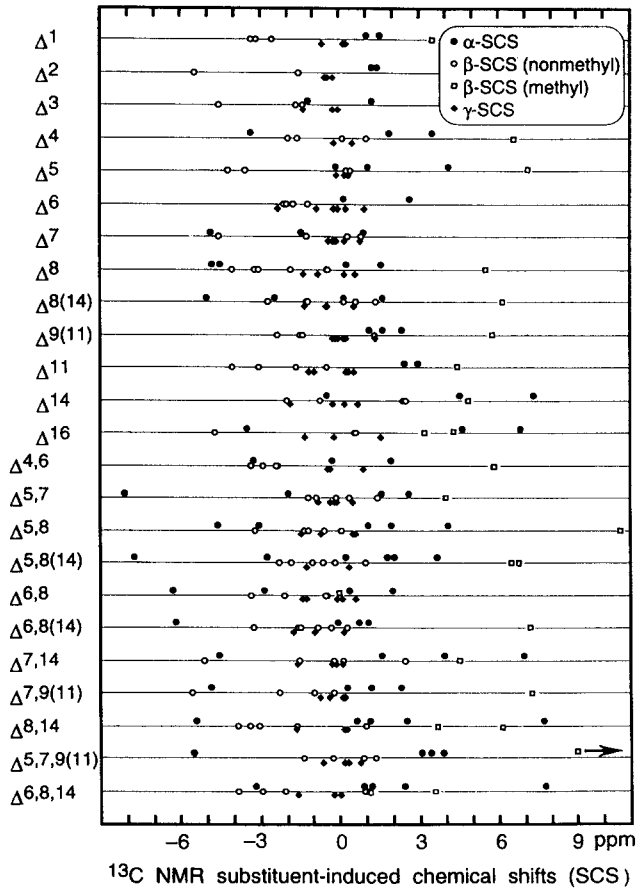

Fig. 7. Effect of unsaturation in the steroid nucleus on ^{13}C chemical shifts. SCS are calculated from chemical shifts of free sterols in Tables 4–6 except for Δ^1, Δ^2, Δ^3, $\Delta^{9(11)}$, and Δ^{11} (calculated from data in ref. 43), and Δ^{16} (calculated as described in footnote 7). Symbols for the α and γ SCS are offset slightly above and below each line corresponding to the β SCS. The β SCS for C-19 of the $\Delta^{5,7,9(11)}$ sterol is off scale at 18.1 ppm (indicated by arrow).

over most data given previously for unsaturated sterols, should facilitate structure determination of new sterols and permit higher confidence in signal assignments established by comparisons. As discussed below, the precision is also very useful in the identification of sterols in a complex mixture.

The spectral dispersion of resolution-enhanced ^1H NMR spectra at 500 MHz permitted the determination of ^1H-^1H coupling constants (Tables 7–9) from many first-order multiplets of the unsaturated sterols. Additional couplings were calculated from resonances isolated by NOE or saturation difference spectroscopy (18, 19). Couplings of lower precision were estimated from f_2 cross sections of HSQC spectra and from 1D spectra showing limited second-order effects. Distortion from second-order effects precluded the reliable determination of J values in many spin systems, including nearly all the methylene side-chain protons. The extent of strong coupling between a pair of protons can be gauged by the ratio of their chemical shift difference to their mutual coupling constant ($\Delta\nu/J$), and proton pairs having values <3 are noted in Tables 7–9. Much of this strong coupling will not be eliminated at field strengths less than several GHz.

The coupling data in Tables 7–9 reflect the conformational diversity of unsaturated sterols. Values of $J_{11\alpha-12\alpha}$ and $J_{11\beta-12\beta}$ are 4 Hz when ring C is a chair but increase to 7–8 Hz in the 13β sofa conformation of the $\Delta^{5,8}$ sterol. In a 12α-sofa conformation, observed for the $\Delta^{8(14)}$ and $\Delta^{5,8(14)}$ sterols, $J_{11\alpha-12\alpha}$ and $J_{11\beta-12\beta}$ differ only slightly from values for a chair conformation. However, a 12α-sofa can be recognized by the distinctive values for $J_{9\alpha-11\alpha}$ and $J_{9\alpha-11\beta}$ (7 Hz and 10 Hz), which have been also been observed in $\Delta^{8(14)}$-15-ketosterols (14). The various conformations for ring D can similarly be distinguished according to values of $J_{15\alpha-16\beta}$, $J_{15\beta-16\alpha}$, $J_{16\alpha-17\alpha}$, and $J_{16\beta-17\alpha}$. The experimental couplings in Tables 7–9 are generally compatible with ring conformations derived from molecular mechanics (Table 10) as judged by agreement with couplings predicted by an extended Karplus relationship (50).

Correlation of NMR chemical shifts with structure

Detailed understanding of the effect of functional groups on NMR chemical shifts is extremely valuable in structure elucidation, and exceptions to established patterns may suggest errors in signal or structure assignments or an unanticipated conformation. A simple measure of the effect of unsaturation is given by substituent-induced chemical shifts (SCS), which have been calculated for each carbon atom of each unsaturated sterol in Tables 4–6 by subtracting the corresponding chemical shift of cholestanol or cholestanol acetate.[6] In addition to these SCS of unusually high precision, ^{13}C SCS of lower precision have been calculated for Δ^1, Δ^2, Δ^3, $\Delta^{9(11)}$, and Δ^{11} from data reported by Eggert and Djerassi (43) and for Δ^{16} from our data.[7] The distribu-

[6]A tabulation of the ^1H and ^{13}C SCS and tables indicating the degree of additivity of the SCS are available upon request from the authors. Also available are tables of acetylation shifts for all ^1H and ^{13}C resonances. The latter tables reveal subtle long-range effects of acetylation (small upfield shifts for H-6β, H-7β, H-8β, H-11α, H-11β, H-12β, H-15β, H-16β, H-18 and H-20 and small downfield shifts for H-7α, H-9α, H-14α, H-16α, H-17α, and H-22S).

[7]SCS were calculated from NMR data for (25*R*)-26-hydroxycholesterol (51) and (25*R*)-cholesta-5,16-diene-3 β,26-diol (δ 11.85 and δ 16.13 for C-18). The latter sterol was prepared by conversion of (25*R*)-3β,26-bis(*tert*-butyldimethylsily- loxy)cholest-5-en-16β-ol (51) to its 16β-mesylate, followed by reduction with $LiAlH_4$ under conditions analogous to those described for $\Delta^{5,22}$ sterols (52). (25*R*)-3β,26-bis(*tert*-butyldimethylsilyloxy)cholesta- 5,16-diene, obtained as a byproduct of the reduction, was hydrolyzed to the Δ^{16}-3β,26-diol (Y. Ni, W. K. Wilson, and G. J. Schroepfer, Jr., unpublished results). It should be noted that the Δ^{16} bond markedly alters the C13-C17-C20-C22 dihedral angle, and this effect may also influence the SCS for C-18.

tion of ^{13}C SCS at α, β, and γ positions is shown in Fig. 7 for each of the unsaturated sterols.

Several attempts have been made to correlate double-bond substituent effects with steroid structure (43, 53–55). However, simple attempts to predict α, β, γ, and δ SCS for steroidal olefins are hampered by complications arising from variable conformational (Table 10) and branching patterns and from increased transmission of electric field effects through π electron systems. One of the best empirical correlations for steroidal olefins relates the introduction of a double bond to a downfield shift of several ppm for homoallylic axial methyl carbons (43, 53–55). These downfield shifts are observed for C-18 and C-19 of steroids as well as for ring substituents, such as 6β-methyl (56). Eggert and Djerassi (43) noted one exception, in which introduction of a Δ^{16} double bond in a C_{19} steroid led to only a 0.4 ppm upfield shift for C-18, a finding that was stated to be in agreement with predictions in ref. 54. However, our SCS data for C-18 of a Δ^{16} C_{27} sterol[7] show a normal downfield shift of 4.3 ppm. Our other SCS data are in accord with the empirical rule except for the $\Delta^{6,8}$ and $\Delta^{6,8,14}$ sterols, for which the C-19 SCS is 0.0 ppm and 1.0 ppm, respectively. It has been pointed out (33) that the usual downfield shift may be markedly reduced for conjugated homoannular dienes, such as $\Delta^{5,7}$ and $\Delta^{6,8}$ sterols. Although this proposal holds for cholesta-2,4,6-triene (δ 15.4 for C-19), it fails for $\Delta^{2,4}$ dienes (δ 20.0 for C-19) (W. K. Wilson and G. J. Schroepfer, Jr., unpublished results) and seems incompatible with the 8.1-ppm SCS for C-19 in 3β-hydroxy-5α-cholesta-8(14),9(11)-dien-15-one (57) or the remarkably large SCS of 18 ppm for C-19 in the $\Delta^{5,7,9(11)}$ sterol.

The foregoing example illustrates how empirical rules for substituent effects may be of limited predictive value, especially when the scope of the rule is unknown owing to unsound or nonexistent theoretical underpinnings. Quantum mechanical calculations may eventually furnish reliable predictions of NMR chemical shifts. Until then, as Eggert and Djerassi (43) have pointed out, structure elucidation and signal assignments for unsaturated sterols should be based on experimental values from model systems rather than on predictions derived from empirical correlations. The highly precise ^{13}C NMR data presented herein should be valuable for this purpose.

In contrast to the ^{13}C SCS data, ^{1}H NMR SCS fell into regular patterns. As shown in **Fig. 8,** introduction of unsaturation generally led to downfield shifts, the largest being for allylic protons (0.3 to 1.7 ppm), followed by homoallylic protons (mainly <0.5 ppm) and more distant protons (mainly <0.2 ppm). The pseudo-axial allylic protons were usually shifted farther downfield than pseudo-equatorial protons, and most of the large homoallylic shifts were for axial or pseudo-axial protons. Several long-range effects of unsaturation were notable, such as the effect of $\Delta^{8(14)}$ on H-22S (ca. 0.09 ppm in **7a, 7b, 12a, 12b, 15a,** and **15b**) and the effect of Δ^{14} on H-1β (0.07 ppm in **8a** and **8b**). Unsaturation at all positions except Δ^{4} had a small but measurable effect on the terminal side-chain protons (H-25, H-26, H-27), which are up to 12 bonds away from the π electrons. By contrast, unsaturation in the steroid nucleus had virtually no measurable effect on the ^{13}C shieldings of the terminal side-chain carbons.

The ^{1}H SCS derived from Tables 1–3 conformed to additivity relationships noted in past NMR studies of steroids (40, 58). The non-olefinic SCS for dienes and trienes were reasonably well predicted as the sum of the SCS for the individual double bonds, even for conjugated systems. The errors from these predictions constituted only about 15% of the total of all non-olefinic SCS. The present ^{1}H SCS are in remarkably good agreement

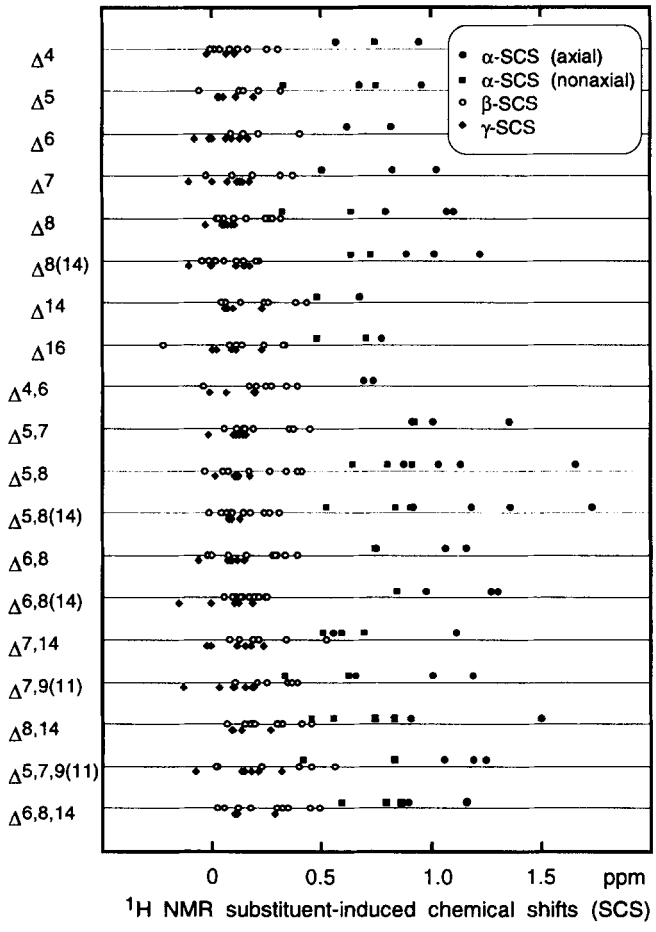

Fig. 8. Effect of unsaturation in the steroid nucleus on ^{1}H chemical shifts. SCS are calculated from chemical shifts of free sterols in Tables 1–3 except for Δ^{16} (calculated as described in footnote 7). Symbols for the α and γ SCS are offset slightly above and below each line corresponding to the β SCS. The axial α-SCS include pseudo-axial protons and H-15β.

with data compiled in 1963 by Zürcher (58) for H-18 and H-19.

Use of NMR for identification of unsaturated sterols

The structure of a sterol can be established unequivocally by standard X-ray crystallographic or NMR methods (10), both of which typically require at least a milligram of material. Other approaches involving various forms of chromatography, other spectral methods (MS, UV, IR), and chemical conversion to compounds of known structure can be carried out with much lower amounts of sample. However, meaningful application of these methods to unsaturated sterols depends critically upon the availability of a comprehensive set of authentic standards or valid and reproducible chromatographic and spectral data for a wide range of sterols. Extensive combinations of chromatographic and spectral techniques are almost invariably required for assignment of structure with any measure of confidence.

This situation derives largely from the insensitivity of most techniques to double bond position. Standard TLC, HPLC, and GC depend upon differences in polarity, hydrophobicity, or volatility, and these properties are frequently very similar among unsaturated sterols. Many pairs of unsaturated sterols can be resolved using these relatively rapid and sensitive chromatographic techniques, but high chromatographic efficiency is needed, and differences in retention values are frequently small (31, 59). Chromatography on silver ion-impregnated supports can provide effective separations of unsaturated C_{27} sterols (12, 60, and references cited therein), but these relatively time-consuming methods have not been widely applied by others. Many conjugated dienes have distinctive UV spectra, but standard UV methods are generally not reliable for identifying monoenes or non-conjugated dienes. Mass spectrometry, an extremely sensitive and powerful technique, also has limited ability to differentiate among unsaturated sterols, pairs of which frequently have identical molecular ions and highly similar fragmentation patterns in electron impact spectra. For example, the $\Delta^{5,7}$ and $\Delta^{6,8}$ trimethylsilyl ethers (61) show essentially identical mass spectra. Even when small differences in fragmentation tendencies are noted, it is difficult to develop such observations into a reliable method of identification because of day-to-day and instrument-to-instrument variations in relative ion abundances.

^{1}H and ^{13}C NMR spectra, by contrast, are highly sensitive to double bond position. Introduction of unsaturation leads to distinctive olefinic signals and markedly affects the position of other resonances up to several bonds away (Figs. 7 and 8). Moreover, NMR spectra of sterols furnish a fingerprint far more distinctive than that from any combination of chromatographic or other spectroscopic methods except X-ray crystallography. The NMR data can be efficiently summarized as a set of chemical shifts and coupling constants (Tables 1–9), which, unlike actual spectra, are

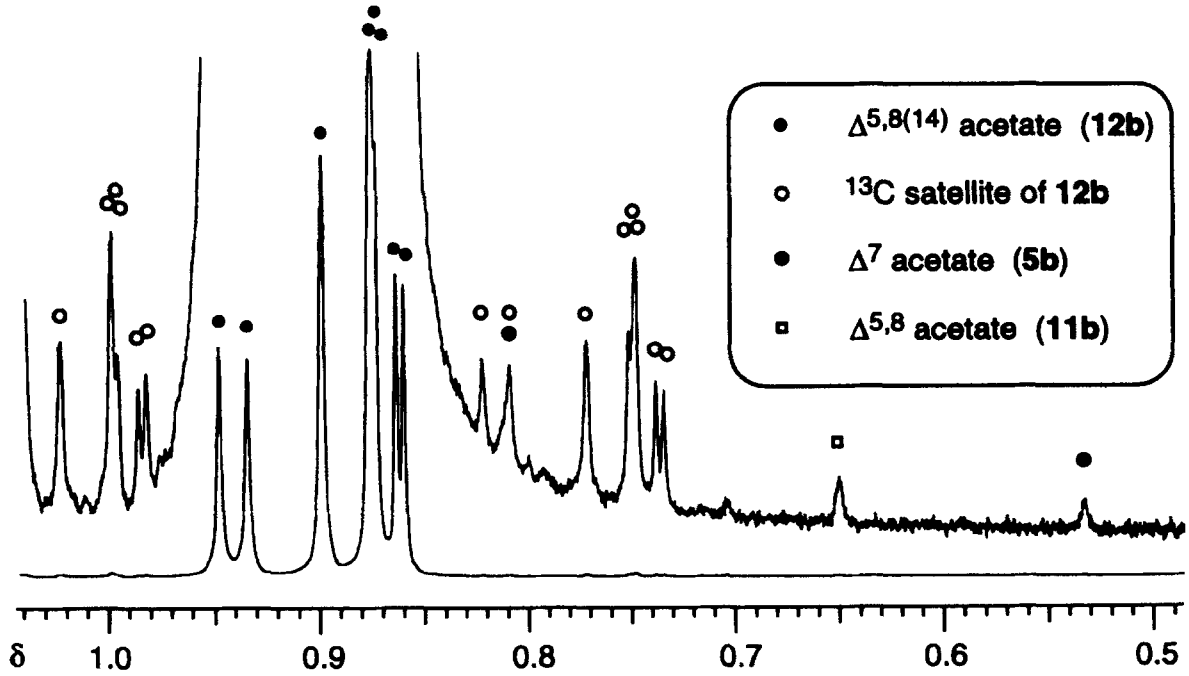

Fig. 9. Methyl region of the ^{1}H NMR spectrum of the $\Delta^{5,8(14)}$ acetate **12b** (2.7-s acquisition time; no apodization). The vertical scale expansions show ^{13}C satellites and other signals probably corresponding to trace impurities of **5b** (0.1%) and **11b** (0.2%).

directly comparable with those of unknowns without compensation for differences in magnetic field strength.

In practice, identification is normally based on a subset of the data in Tables 1–9, depending on the certainty required. An HSQC spectrum or a simple unassigned ^{13}C spectrum matching values of a sterol in Tables 4–6 to ±0.03 ppm provides virtually unambiguous identification. However, an overnight ^{13}C or HSQC spectrum requires 700 μg of sterol on our spectrometer[8] or 100 μg with a suitable microprobe (62–65), and availability of material or spectrometer time may preclude such analysis. Assigned ^{1}H NMR chemical shifts and a partial set of coupling constants compatible with values in Tables 1–3 and 7–9 also afford high reliability of identification. These data can be obtained from 1D and COSYDEC spectra in 1 h of spectrometer time from 200 μg of sterol, and a microprobe might reduce this amount to the low microgram range. One step lower in reliability, a high quality ^{1}H NMR spectrum at 500 MHz will nevertheless usually define the sterol structure uniquely. This requires only a 10-min acquisition on a 10-μg sample or an overnight acquisition on a 1-μg sample and careful comparison of chemical shifts and coupling constants with those in Tables 1–3 and 7–9. A spectrum showing only the methyl signals requires 10–20 min of spectrometer time for a 1-μg sample (Fig. 5), and low nanogram levels might be sufficient with appropriate microprobe instrumentation (62–66). The methyl shieldings alone may not uniquely define a sterol structure, although, because of their high sensitivity to double-bond locations, they will distinguish all pairs of unsaturated sterols in Fig. 1. The power of NMR to discriminate among sterol structures is greatly reduced at lower precision, such as the ±0.01 ppm precision commonly found in the literature for proton data.

The ability to collect highly informative NMR data relatively quickly from samples of 1 μg or less suggests applications in the analysis of trace amounts of sterols of biological origin. In addition to adequate sensitivity and high reliability of identification, NMR has several other desirable features. Because NMR chemical shifts are highly reproducible, no internal standards or authentic samples are needed. Under conditions recommended herein, NMR is non-destructive, and no derivatization is required. Sample preparation consists simply of dissolving the solvent-free sample in $CDCl_3$. However, for routine analyses of a highly repetitive nature, NMR is unlikely to supplant HPLC, LC-MS, GC, and GC-MS, techniques that feature better sensitivity of detection, lower instrument cost, and greater ease of automation.

[8]The present work was done on a 6-year-old 500 MHz spectrometer with an inverse-geometry 5-mm probe, which currently shows a signal-to-noise ratio of ~350 with 0.1% ethylbenzene in $CDCl_3$. This sensitivity level is surpassed by current 400-MHz instruments, and much greater sensitivity can be obtained on higher field instruments, especially in conjunction with a microsample probe (62–66).

NMR analysis of mixtures of unsaturated sterols

^{1}H and ^{13}C NMR are useful for determining the composition of mixtures of unsaturated sterols. This requires both identification and quantitation of the components. ^{13}C NMR spectra are highly informative because typically numerous signals of each component are resolved from other signals, thus making identification of minor components highly reliable. However, low sensitivity and imprecise quantitation are limiting factors. ^{1}H NMR spectra offer much higher sensitivity but at the expense of extensive signal overlap. At 500 MHz, distinctive well-resolved signals are likely to be found in the methyl (δ 0.5–1.2) and the olefinic (δ 4.5–6.5) regions. In unsaturated C_{27} sterols, the downfield portion of the methyl region is cluttered by multiplets and signals of the side-chain methyls, but the upfield region (δ 0.5–0.84) usually contains only singlets corresponding to H-18 or H-19, whose signals are sensitive to the position of nearby double bonds. The extent of problems with signal overlap depends on lineshapes, apodization, field strength, and the relative intensities of the peaks. At 500 MHz with 1-Hz line broadening, H-18 or H-19 signals differing by 0.005 ppm can be resolved if the intensities are comparable, whereas a separation of ≥0.015 ppm may be needed to resolve a 1% component from peaks of the major sterol. Levels of minor components can be estimated by comparison with ^{13}C satellites of the predominant sterol.[2]

Olefinic signals are dispersed over a large region (>1 ppm), and their shieldings and distinctive coupling patterns allow reliable identification of components. Overlap is sometimes a problem because olefinic multiplets, which are much wider than methyl singlets, are often clustered in the δ 5.3–5.5 region. Usually overlap is incomplete, and identification of components and an estimate of their relative amounts can be derived from resolved portions of the multiplet. In addition to the methyl and olefinic regions, the allylic region (δ 2.0–2.8) and the acetate methyl region (δ 2.02–2.06) are sometimes informative.

NMR analysis according to the preceding criteria was extremely helpful in determining the composition of the complex diene mixtures obtained in the synthesis of the $\Delta^{6,8}$ benzoate **14c** and in establishing the purity of sterols. Analyses can be done on 1-μg quantities (Fig. 5), and trace sterols may be identifiable in the presence of a major sterol component (**Fig. 9**), a situation often encountered with partially purified samples of biological origin. However, signals of the overwhelming sterol component obscure a significant portion of the methyl region, and their ^{13}C satellites obscure additional re-

gions. Usually at most one signal of a trace component will be detectable in the methyl region, and its identification may be no more reliable than those based on HPLC or GC retention times. For example, minor contaminants in commercial 7-dehydrocholesterol (**10a**) showed ^{1}H singlets at δ 0.545 and 0.566, shieldings apparently corresponding to vitamin D_3 (**22a**) and the $\Delta^{5,7,9(11)}$ triene (**21a**). Subsequent analysis of a more concentrated sample revealed minor olefinic signals confirming the presence of **21a** but showing no olefinic resonances corresponding to **22a.**

The criteria for analyzing mixtures by NMR were also used to estimate purities of unsaturated sterols. For example, vertical-scale expansions of the methyl region of the $\Delta^{5,8(14)}$ acetate **12b** (Fig. 9) show ^{13}C satellites (0.55% intensity of the main peak) and impurity signals at δ 0.650 (0.2%) and δ 0.533 (0.1%), apparently corresponding to the $\Delta^{5,8}$ and Δ^7 acetates. Another Δ^7 signal at δ 0.810 is largely hidden under a ^{13}C satellite. Other potential trace impurities (≤0.2%) may be undetectable, with any olefinic signals lost in the noise and all their methyl signals hidden under other peaks. However, the likelihood of detecting a larger sterol impurity (≥0.5%) is much greater because its signals will protrude conspicuously above isochronous ^{13}C satellites and noise. Estimating purity levels of unsaturated sterols by ^{1}H NMR requires narrow lines, high sensitivity (preferably an inverse detection probe), high field strength for good spectral dispersion, and attention to apodization. Very mild Gaussian apodization gives narrow lines and good sensitivity but with some intensity distortion. Usually we prefer no apodization in the methyl region and exponential line broadening (1 Hz) in the olefinic region. Under these conditions, a 5-min ^{1}H NMR spectrum of a 1-mg sample of any sterol in Fig. 1 would reveal a 1% impurity of any other sterol in that figure.

Sample preparation and spectrometer operating conditions

All NMR spectra described herein were measured in $CDCl_3$, an inexpensive solvent available in high isotopic purity. Sterols dissolve easily in $CDCl_3$, and the past popularity of this solvent facilitates comparisons with previous sterol NMR data. A potential problem is the stability of sterols in $CDCl_3$ solution, especially aged $CDCl_3$, which may contain DCl and phosgene. Although $CDCl_3$ may be especially prone to decomposition because it contains no stabilizers, $CDCl_3$ aged for a year or more under air at room temperature is not infrequently used in routine analyses, apparently without harmful effects in most cases. Using relatively fresh $CDCl_3$, we did not detect any decomposition during the NMR analyses of the unsaturated sterols. In a more rigorous experiment, the stability of several sterols in $CDCl_3$ solution was monitored over the period of a week by comparing ^{1}H NMR signal intensities with those of a stable internal standard (5α-cholestane). The results, summarized in Fig. 4, showed significant decomposition only for $\Delta^{5,7}$ and $\Delta^{6,8}$ isomers, and then only after several days. Both the $\Delta^{5,7}$ (67) and $\Delta^{6,8}$ (37) sterols are known to be rather labile. These results suggest that unsaturated sterols will undergo at most slight decomposition (1–2%) during overnight NMR analysis.

Lower stability might be anticipated for sterols in $CDCl_3$ from sealed ampules because of possible thermal decomposition of $CDCl_3$ vapors during the sealing process. Experiments analogous to those in Fig. 4 for 1-mg amounts of the $\Delta^{5,7}$ acetate **10b** in $CDCl_3$ from a sealed ampule showed variable results, from virtually no decomposition after 17 h to 7% decomposition after 4 h and ~15% decomposition after 30 h. Far more decomposition was observed with samples in the low microgram range. For example, a mixture of 1 μg each of three unsaturated sterol acetates (**3b, 5b,** and **10b**) with 5α-cholestane showed the correct intensities for the methyl signals in $CDCl_3$ from screw-cap bottles but either partial or (in most cases) complete decomposition of the unsaturated sterols in $CDCl_3$ from sealed ampules (Fig. 5). Although a few samples of $CDCl_3$ from sealed ampules gave marginally acceptable stability, most, which were distributed among three lots from two different suppliers, led to total decomposition of the unsaturated sterols. Based on these experiences, we recommend using $CDCl_3$ only from screw-cap bottles. Each new lot should routinely be tested with a mixture of 1 μg each of 5α-cholestane and **3b** or **10b,** especially before use with irreplaceable samples.

Unlike GC-MS and HPLC, ^{1}H NMR analysis requires the exclusion of all solvent containing hydrogen atoms. Except for hexane (δ 0.88, t) and dichloromethane (δ 5.30, s), most solvents do not show signals in the important methyl and olefinic regions and may be tolerated in small amounts. Hexane of high purity is a useful solvent for storing microgram and nanogram amounts of unsaturated sterols. Evaporation of such solutions in a stream of nitrogen followed by a few minutes exposure to high vacuum almost completely eliminated hexane signals in spectra of 1-μg samples. Some spectra showed minor amounts of methyl silicone (δ 0.069, s), long-chain alkyl contaminants (δ 1.26, br s; δ 0.88, t, 7 Hz), and acetone (δ 2.17), but these signals did not interfere with spectral interpretation or quantitation.

Large solvent signals from residual $CHCl_3$ (δ 7.26, s) in $CDCl_3$ and HDO (δ ~1.56, s) from adventitious moisture are almost unavoidable. Although these signals fall

in relatively unimportant spectral regions, their large size leads to unnecessarily low receiver gain settings that may reduce sensitivity. Our attempts to reduce the size of these peaks by using highly enriched $CDCl_3$ in sealed ampules usually led to decomposition of the unsaturated sterols. Use of 99.8% $CDCl_3$ from screw-cap bottles with modest precautions to exclude moisture led to receiver gains only one step lower than with the highly enriched solvent and little difference in sensitivity. Because of the long relaxation time for $CHCl_3$, introduction of 16 dummy scans prior to beginning the acquisition dramatically reduced the size of the $CHCl_3$ signal and sometimes allowed an increase in receiver gain. The HDO signal, which is less readily saturated than the $CHCl_3$ peak, could be presaturated by low-power irradiation prior to each pulse, but possible NOE effects from multiplets near the HDO peak (e.g., H-11β) might distort the intensities for H-18 and H-19.

Maximizing sensitivity for microgram quantities of sterols requires attention to NMR operating conditions. Improvements in sensitivity by reduction of the sample volume from the usual 500 μl to 350 μl were marginal for ^1H spectra, probably because of lineshape distortions due to magnetic susceptibility effects, which cannot be corrected entirely by shimming adjustments without magic angle spinning (65) or matched susceptibility plugs. Sensitivity can also be improved by decreasing the acquisition time and adjusting the tip angle according to the equation $\cos \alpha_E = \exp(-T_r/T_1)$, where α_E is the optimal tip angle (Ernst angle), T_r is the time between pulses (here the acquisition time), and T_1 is the relaxation time. The relaxation times for nearly all the aliphatic protons in the steroid nucleus were found to be 0.5–1.0 s, corresponding to a tip angle of ~70–80° for a 1-s acquisition time. Similar values were found by trial-and-error adjustment of these parameters for optimal sensitivity. For samples of ~1 μg, a 1-s acquisition time with 1-Hz exponential line broadening was used. Resolution was improved somewhat for samples of ~10 μg by increasing the acquisition time to 1.5 s and using 0.5-Hz line broadening.

Summary

We have presented complete ^1H and ^{13}C NMR chemical shifts and ^1H-^1H coupling constants for a large collection of unsaturated sterols, including many of biological interest. The high precision and reproducibility of these data markedly enhance their value for the identification of unknown sterols by chemical shift comparisons. With numerous highly precise measurements correlating directly to chemical structure, identification of unknowns by NMR is typically far more dependable than by HPLC, GC, or GC-MS, methods that by themselves have relatively low discriminating power among unsaturated sterols. Unequivocal identification of 1-mg quantities of unsaturated sterols can be made from ^{13}C or HSQC spectra, and 1-μg quantities can be identified reliably by careful comparison of the 500-MHz ^1H spectrum with data presented herein. Existing microprobe technology should lower these detection limits by an order of magnitude. Components of mixtures of unsaturated sterols can also be identified by NMR, even in rather complex mixtures encompassing a wide range of concentrations, although identification of minor components at nanogram levels may be of modest reliability. NMR is a non-destructive method, and unsaturated sterols are stable under appropriate conditions of analysis. Unlike chromatographic retention times and relative ion intensities in mass spectra, NMR chemical shifts and coupling constants for sterols are highly reproducible from one instrument to another under standardized operating conditions. The sensitivity and resolution needed to obtain suitable NMR spectra are commonly available on higher field spectrometers. The foregoing considerations indicate that NMR should be an extremely valuable technique in the identification of unsaturated sterols isolated in trace amounts from biological materials.

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REFERENCES

1. Schroepfer, G. J., Jr. 1982. Sterol biosynthesis. *Annu. Rev. Biochem.* **51:** 555–585.
2. Echevarria, F., R. A. Norton, W. D. Nes, and Y. Lange. 1990. Zymosterol is located in the plasma membrane of cultured human fibroblasts. *J. Biol. Chem.* **265:** 8484–8489.
3. Buttke, T. M., and T. M. Folks. 1992. Complete replacement of membrane cholesterol with 4,4′,14-trimethyl sterols in a human T cell line defective in lanosterol demethylation. *J. Biol. Chem.* **267:** 8819–8826.
4. Izumi, A., F. D. Pinkerton, S. O. Nelson, J. S. Pyrek, P. J. G. Neill, J. H. Smith, and G. J. Schroepfer, Jr. 1993. Inhibitors of sterol synthesis. Submicromolar 14α-ethyl-5α-cholest-7-ene-3β,15α-diol causes a major modification of the sterol composition of CHO-K1 cells and a marked change in cell morphology. *J. Lipid Res.* **35:** 1251–1266.
5. Johnson, W. J., R. T. Fisher, M. C. Phillips, and G. H. Rothblat. 1995. Efflux of newly synthesized cholesterol and biosynthetic sterol intermediates from cells. *J. Biol. Chem.* **270:** 25037–25046.
6. Tint, G. S., M. Irons, E. R. Elias, A. K. Batta, R. Frieden, T. S. Chen, and G. Salen. 1994. Defective cholesterol

biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330:** 107–113.

7. Tint, G. S., M. Seller, R. Hughes-Benzie, A. K. Batta, S. Shefer, D. Genest, M. Irons, E. Elias, and G. Salen. 1995. Markedly increased tissue concentrations of 7-dehydrocholesterol combined with low levels of cholesterol are characteristic of the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 89–95.
8. Honda, A., G. S. Tint, G. Salen, A. K. Batta, T. S. Chen, and S. Shefer. 1995. Defective conversion of 7-dehydrocholesterol to cholesterol in cultured skin fibroblasts from Smith-Lemli-Opitz syndrome homozygotes. *J. Lipid Res.* **36:** 1595–1601.
9. Byskov, A. G., C. Y. Andersen, L. Nordholm, H. Thogersen, X. Guoliang, O. Wassmann, J. V. Andersen, E. Guddal, and T. Roed. 1995. Chemical structure of sterols that activate oocyte meiosis. *Nature.* **374:** 559–562.
10. Wilson, W. K., and G. J. Schroepfer, Jr. 1992. ^{1}H and ^{13}C NMR spectroscopy of sterols. *In* Molecular Structure and Biological Activity of Steroids. M. Bohl and W. L. Duax, editors. CRC Press, Boca Raton, FL. 33–90.
11. Siddiqui, A. U., W. K. Wilson, S. Swaminathan, and G. J. Schroepfer, Jr. 1992. Efficient preparation of steroidal 5,7-dienes of high purity. *Chem. Phys. Lipids.* **63:** 115–129.
12. Pascal, R. A., Jr., C. L. Farris, and G. J. Schroepfer, Jr. 1980. Sterol synthesis: medium-pressure chromatography of C_{27} sterol precursors of cholesterol on alumina-silver nitrate columns. *Anal Biochem.* **101:** 15–22.
13. Kirk, D. N., and H. C. Toms. 1991. ω_1-Decoupled ^{1}H homonuclear shift-correlated nuclear magnetic resonance spectroscopy (COSYDEC) applied to steroids. *Steroids.* **56:** 195–200.
14. Siddiqui, A. U., N. Gerst, L. Kim, F. D. Pinkerton, A. Kisic, W. K. Wilson, and G. J. Schroepfer, Jr. 1994. Inhibitors of sterol synthesis. Effects of a 7α-alkyl analog of 3β-hydroxy-5α-cholest-8(14)-en-15-one on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured mammalian cells and on serum cholesterol levels and other parameters in rats. *Chem. Phys. Lipids.* **70:** 163–178.
15. States, D. J., R. A. Haberkorn, and D. J. Ruben. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.* **48:** 286–292.
16. Bodenhausen, G., and D. J. Ruben. 1980. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem. Phys. Lett.* **69:** 185–189.
17. Marion, D., M. Ikura, R. Tschudin, and A. Bax. 1989. Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J. Magn. Reson.* **85:** 393–399.
18. Ley, S. V., A. J. Whittle, and G. E. Hawkes. 1983. Stereochemistry of a 5,5,10-trimethyl-2-decalone by proton nuclear magnetic resonance: a new application of INDOR difference spectroscopy. *J. Chem. Res. (S)* **8:** 210–211.
19. Bauer, C., and R. Freeman. 1985. Decomposition of proton NMR spectra into individual spin multiplets. *J. Magn. Reson.* **61:** 376–381.
20. Dykstra, R. W. 1993. A technique which eliminates the irradiation spike in homonuclear decoupling experiments. *J. Magn. Reson., Ser. A* **102:** 114–115.
21. Kudo, K., G. T. Emmons, E. W. Casserly, D. P. Via, L. C. Smith, J. S. Pyrek, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chromatography of acetate derivatives of oxygenated sterols. *J. Lipid Res.* **30:** 1097–1111.
22. Tsuda, M., and G. J. Schroepfer, Jr. 1979. Carbon-13 nuclear magnetic resonance studies of C_{27} sterol precursors of cholesterol. *J. Org. Chem.* **44:** 1290–1293.
23. Paliokas, A. M., and G. J. Schroepfer, Jr. 1968. Stereospecificity in the enzymatic conversion of Δ^7-cholesten-3β-ol to 7-dehydrocholesterol. *J. Biol. Chem.* **243:** 453–464.
24. Schroepfer, G. J., Jr., and I. D. Frantz, Jr. 1961. Conversion of Δ^7-cholestenol-4-C^{14} and 7-dehydrocholesterol-4-C^{14} to cholesterol. *J. Biol. Chem.* **236:** 3137–3140.
25. Lee, W-H., R. Kammereck, B. N. Lutsky, J. A. McCloskey, and G. J. Schroepfer, Jr. 1969. Studies on the mechanism of the enzymatic conversion of Δ^8-cholesten-3β-ol to Δ^7-cholesten-3β-ol. *J. Biol. Chem.* **244:** 2033–2040.
26. Siddiqui, A. U., W. K. Wilson, E. J. Parish, N. Gerst, F. D. Pinkerton, and G. J. Schroepfer, Jr. 1994. Inhibitors of sterol synthesis. Synthesis and spectral properties of 3β-hydroxy-5α-cholestan-15-one and its 14β-epimer and their effects on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Chem. Phys. Lipids.* **74:** 1–15.
27. Lee, W-H., B. N. Lutsky, and G. J. Schroepfer, Jr. 1969. 5α-Cholest-8(14)-en-3β-ol, a possible intermediate in the biosynthesis of cholesterol. *J. Biol. Chem.* **244:** 5440–5448.
28. Schroepfer, G. J., Jr., E. J. Parish, and A. A. Kandutsch. 1988. Inhibitors of sterol biosynthesis. Synthesis and activities of ring C oxygenated sterols. *Chem. Phys. Lipids.* **46:** 147–154.
29. Lutsky, B. N., J. A. Martin, and G. J. Schroepfer, Jr. 1971. Studies of the metabolism of 5α-cholesta-8,14-dien-3β-ol and 5α-cholesta-7,14-dien-3β-ol in rat liver homogenate preparations. *J. Biol. Chem.* **246:** 6737–6744.
30. Taylor, U. F., A. Kisic, R. A. Pascal, Jr., A. Izumi, M. Tsuda, and G. J. Schroepfer, Jr. 1981. Sterol synthesis: a simple method for the isolation of zymosterol (5α-cholesta-8,24-dien-3β-ol) from yeast and spectral properties of zymosterol. *J. Lipid Res.* **22:** 171–177.
31. Wilson, W. K., and G. J. Schroepfer, Jr. 1988. Acid-catalyzed isomerization of 7-dehydrocholesterol benzoate. A revised mechanism and an improved synthetic procedure. *J. Org. Chem.* **53:** 1713–1719.
32. Fisher, R. T., F. A. Stephenson, A. Shafiee, and F. Schroeder. 1984. $\Delta^{5,7,9(11)}$-Cholestatrien-3β-ol: a fluorescent cholesterol analogue. *Chem. Phys. Lipids.* **36:** 1–14.
33. Parish, E. J., M. Tsuda, and G. J. Schroepfer, Jr. 1979. Synthesis and spectral properties of 5α-cholesta-6,8,14-trien-3β-ol. *Chem. Phys. Lipids.* **25:** 111–124.
34. Kircher, H. W., and F. U. Rosenstein. 1987. Preparation of desmosterol from (20*S*,22*R*,*S*)-3β-acetoxychola-5,23-dien-22-ol. *J. Org. Chem.* **52:** 2586–2588.
35. Kircher, H. W., and F. U. Rosenstein. 1982. Preparation of some unsaturated side-chain derivatives of cholesterol. *J. Org. Chem.* **47:** 1722–1724.
36. Anastasia, M., A. Fiecchi, and G. Galli. 1981. Synthesis of cholesta-5,8-dien-3β-ol. *J. Org. Chem.* **46:** 3421–3422.
37. Windaus, A., O. Linsert, and H. J. Eckhardt. 1938. Über Iso-dehydro-cholesterin (Δ_6,Δ_8-Cholestadien-3-ol). *Justus Liebigs Ann. Chem.* **534:** 22–41.
38. Hunziker, F., F. X. Müllner, K. G. Reuter, and H. Schaltegger. 1955. Zur Dehydrobromierung von 7-Brom-cholesteryl-benzoat: abnorme Produkte. *Helv. Chim. Acta.* **38:** 1316–1328.
39. Yang, D., X. Xu, and C. Ye. 1992. Application of HMQC to the measurement of J(H,H) homonuclear coupling constants. *Magn. Reson. Chem.* **30:** 711–715.

40. Kirk, D. N., H. C. Toms, C. Douglas, K. A. White, K. E. Smith, S. Latif, and R. W. P. Hubbard. 1990. A survey of the high-field ^{1}H NMR spectra of steroid hormones, their hydroxylated derivatives, and related compounds. *J. Chem. Soc., Perkin. Trans.* 2: 1567–1594.
41. Kisic, A., M. Tsuda, R. J. Kulmacz, W. K. Wilson, and G. J. Schroepfer, Jr. 1995. Sphingolipid bases. A revisitation of the *O*-methyl derivatives of sphingosine. Isolation and characterization of diacetate derivatives, with revised ^{13}C nuclear magnetic resonance assignments for D-erythro-sphingosine. *J. Lipid Res.* **36:** 787–803.
42. Wilson, W. K., S. Swaminathan, F. D. Pinkerton, N. Gerst, and G. J. Schroepfer, Jr. 1994. Inhibitors of sterol synthesis. Effects of fluorine substitution at carbon atom 25 of cholesterol on its spectral and chromatographic properties and on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in CHO-K1 cells. *Steroids.* **59:** 310–317.
43. Eggert, H., and C. Djerassi. 1981. Carbon-13 nuclear magnetic resonance spectra of monounsaturated steroids. Evaluation of rules for predicting their chemical shifts. *J. Org. Chem.* **46:** 5399–5401.
44. Batta, A. K., G. S. Tint, S. Shefer, D. Abuelo, and G. Salen. 1995. Identification of 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol) in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 705–713.
45. Joseph-Nathan, P., G. Mejía, and D. Abramo-Bruno. 1979. ^{13}C NMR assignment of the side-chain methyls of C_{27} steroids. *J. Am. Chem. Soc.* **101:** 1289–1291.
46. De Simone, F., A. Dini, E. Finamore, L. Minale, C. Pizza, R. Riccio, and F. Zollo. 1981. Starfish saponins. Part 5. Structure of sepositoside A, a novel steroidal cyclic glycoside from the starfish *Echinaster sepositus. J. Chem. Soc., Perkin Trans.* 1: 1855–1862.
47. Kruk, C., A. W. H. Jans, and J. Lugtenburg. 1985. Two-dimensional INADEQUATE ^{13}C NMR study on vitamin D_3. *Magn. Reson. Chem.* **23:** 267–270.
48. Griffin, J. F., W. L. Duax, and C. M. Weeks. 1984. Atlas of Steroid Structure. Vol. 2 Plenum Press, New York, NY.
49. Altona, C., H. J. Geise, and C. Romers. 1968. Conformation of non-aromatic compounds. Geometry and conformation of ring D in some steroids from X-ray structure determinations. *Tetrahedron.* **24:** 13–32.
50. Haasnoot, C. A. G., F. A. A. M. de Leeuw, and C. Altona. 1980. The relationship between proton-proton NMR coupling constants and substituent electronegativities. An empirical generalization of the Karplus equation. *Tetrahedron.* **36:** 2783–2792.
51. Kim, H-S., W. K. Wilson, D. H. Needleman, F. D. Pinkerton, D. K. Wilson, F. A. Quiocho, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chemical synthesis, structure, and biological activities of (25*R*)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 3β-hydroxy-5αTimes New Roman"ITC-Ne wBaskerville"-cholest-8(14)-en-15-one. *J. Lipid Res.* **30:** 247–261.
52. Ni, Y., H-S. Kim, W. K. Wilson, A. Kisic, and G. J. Schroepfer, Jr. 1993. A revisitation of the Clemmensen reduction of diosgenin. Characterization of byproducts and their use in the preparation of (25R)-26-hydroxysterols. *Tetrahedron Lett.* **34:** 3687–3690.
53. Tsuda, M., and G. J. Schroepfer, Jr. 1979. Nuclear magnetic resonance spectroscopy of sterols. ^{13}C olefinic carbon shieldings in sterols and related cyclic compounds. *Chem. Phys. Lipids.* **25:** 49–68.
54. Beierbeck, H., J. K. Saunders, and J. W. ApSimon. 1977. The semiemperical derivation of ^{13}C nuclear magnetic resonance chemical shifts. Hydrocarbons, alcohols, amines, ketones, and olefins. *Can. J. Chem.* **55:** 2813–2828.
55. Beierbeck, H., and J. K. Saunders. 1976. A reinterpretation of beta, gamma, and delta substituent effects on ^{13}C chemical shifts. *Can. J. Chem.* **54:** 2985–2995.
56. Iida, T., T. Tamura, and T. Matsumoto. 1987. Carbon-13 NMR spectra of some C-6 methylated steroids. *Magn. Reson. Chem.* **25:** 558–560.
57. Siddiqui, A. U., S. Swaminathan, F. D. Pinkerton, N. Gerst, W. K. Wilson, H. Choi, and G. J. Schroepfer, Jr. 1994. Inhibitors of sterol synthesis. Synthesis and spectral properties of derivatives of 3β-hydroxy-25,26,26,26, 27,27,27-heptafluoro-5α-cholest-8(14)-en-15-one fluorinated at carbon 7 or carbon 9 and their effects on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured mammalian cells. *Chem. Phys. Lipids.* **72:** 59–75.
58. Zürcher, R. F. 1963. Protonenresonanzspektroskopie und Steroidstruktur. Die Lage der C-18 und C-19-Methylsignale in Abhängigkeit von den Substituenten am Steroidgerüst. *Helv. Chim. Acta.* **46:** 2054–2088.
59. Thowsen, J. R., and G. J. Schroepfer, Jr. 1979. Sterol synthesis. High-pressure liquid chromatography of C_{27} sterol precursors of cholesterol. *J. Lipid Res.* **20:** 681–685.
60. Schroepfer, G. J., Jr., B. N. Lutsky, J. A. Martin, S. Huntoon, B. Fourcans, W-H. Lee, and J. Vermilion. 1972. Recent investigations on the nature of sterol intermediates in the biosynthesis of cholesterol. *Proc. R. Soc. Lond. B.* **180:** 125–146.
61. Axelson, M. 1991. Occurrence of isomeric dehydrocholesterols in human plasma. *J. Lipid Res.* **32:** 1441–1448.
62. Sharaf, M. H. M., P. L. Schiff, Jr., A. N. Tackie, C. H. Phoebe, Jr., L. Howard, C. Meyers, C. E. Hadden, S. K. Wrenn, A. O. Davis, C. W. Andrews, D. Minick, R. L. Johnson, J. P. Shockcor, R. C. Crouch, and G. E. Martin. 1995. Submicromole structure elucidation: cryptolepicarboline—a novel dimeric alkaloid from *Cryptolepis sanguinolenta. Magn. Reson. Chem.* **33:** 767–778.
63. Shockcor, J. P., R. M. Wurm, I. S. Silver, R. C. Crouch, and G. E. Martin. 1994. Metabolite structure elucidation using NMR micro-/nano-detection. *Tetrahedron Lett.* **35:** 4919–4922.
64. Barbara, T. M. 1994. Cylindrical demagnetization fields and microprobe design in high-resolution NMR. *J. Magn. Reson., Ser. A* **109:** 265–269.
65. Keifer, P. A. 1995. Small volume probes and their applications. (Varian NMR Workshop Series) Varian Associates, Palo Alto, CA.
66. Olson, D. L., T. L. Peck, A. G. Webb, R. L. Magin, and J. V. Sweedler. 1995. High-resolution microcoil ^{1}H-NMR for mass-limited, nanoliter-volume samples. *Science.* **270:** 1967–1970.
67. Fieser, L. F., and M. Fieser. 1959. Steroids. Reinhold Publishing Corp., New York, NY. 158.