24,25-Epoxysterols. Differentiation of 24R and 24S epimers by ^{13}C nuclear magnetic resonance spectroscopy

Gary T. Emmons, William K. Wilson, and George J. Schroepfer, Jr.

Departments of Biochemistry and Chemistry, Rice University, Post Office Box 1892, Houston, TX 77251

Summary The 24R and 24S epimers of the acetates of 24,25-epoxycholesterol and 24,25-epoxylanosterol have been prepared and purified by preparative normal phase high performance liquid chromatography. Neither pair of epimers could be differentiated by ^{1}H NMR. However, the ^{13}C NMR spectra of the epimeric pairs were sufficiently different to permit samples of individual epimers to be assigned as 24R or 24S.—**Emmons, G. T., W. K. Wilson, and G. J. Schroepfer, Jr.** 24,25-Epoxysterols. Differentiation of 24R and 24S epimers by ^{13}C nuclear magnetic resonance spectroscopy. *J. Lipid Res.* 1989. **30:** 133–138.

Supplementary key words 24,25-dihydrolanosterol • cholesterol • GLC-MS • HPLC

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; mp, melting point; I_R and I_S, 24R and 24S epimers of 24,25-epoxycholesterol, respectively; II_R and II_S, 24R and 24S epimers of 24,25-epoxylanosterol, respectively.

Many oxysterols have profound effects on cholesterol biosynthesis (for reviews see 1–3). Among these, the 24R and 24S epimers of 24,25-epoxycholesterol (I_R and I_S) have been reported to inhibit the conversion of 24,25-dihydrolanosterol to cholesterol in rat liver homogenate preparations (4) and to lower the levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in cultured mammalian cells (5, 6). The potential importance of these epoxysterols in the regulation of cholesterol biosynthesis is also suggested by their high affinity binding to the cytosolic oxysterol binding protein (5, 7) and by the demonstration that I_S occurs naturally (5). Whereas no metabolites of I_S have been reported, reduction of the 24R epimer (I_R) to 24(R)-hydroxycholesterol has been observed in rat liver homogenates (8) and in cultured cells (6). Another epoxysterol, 24(S),25-epoxylanosterol (II_S) has been reported to suppress both cholesterol biosynthesis (9) and HMG-CoA reductase activity (6, 10), although it appears to do so only under conditions permitting its further metabolism to 24S,25-epoxycholesterol, I_S (10). Recent work (11, 12), however, indicates that the importance of these 24(S),25-epoxysterols in the normal regulation of cholesterol biosynthesis is not fully established and that further studies of these oxysterols and their metabolites are needed.

These epoxysterols can be prepared by epoxidation of the corresponding Δ^{24}-sterols. The resulting mixture of the C-24 epimers can be difficult to separate. For example, we

and others (10, 13) have been unable to resolve the epimers either as the free sterols or acetates on C_{18} columns routinely used for high performance liquid chromatography (HPLC). However, epimeric mixtures of $\mathbf{I}_R$ and $\mathbf{I}_S$ and of $\mathbf{II}_R$ and $\mathbf{II}_S$ have been separated with a special Vydac C_{18} HPLC column (10, 13). The epimers can also be separated as esters on silica media. Separations have been achieved for the benzoates of $\mathbf{I}_R$ and $\mathbf{I}_S$ by normal phase analytical HPLC (14) and by preparative thin-layer chromatography (TLC) (15) and for the acetates of $\mathbf{II}_R$ and $\mathbf{II}_S$ by preparative TLC (6). In order to resolve the epimers in amounts larger than these reported methods readily permit, we have established conditions for the separation of the acetates of $\mathbf{I}_R$ and $\mathbf{I}_S$ and of $\mathbf{II}_R$ and $\mathbf{II}_S$ by preparative normal phase HPLC. This procedure enabled us to obtain each epimer in quantities sufficient to make reliable ^{13}C nuclear magnetic resonance (NMR) assignments for acetates of $\mathbf{I}_R$, $\mathbf{I}_S$, $\mathbf{II}_R$, and $\mathbf{II}_S$ by one- and two-dimensional NMR techniques. We show here that ^{13}C NMR provides a reliable nondestructive method for identifying the stereochemistry at C-24 of the 24,25-epoxysterols.

EXPERIMENTAL

Lanosterol (Mann Research Laboratories, New York, NY) was recrystallized from methanol. Desmosterol acetate (Steraloids, Wilton, NH), 3-chloroperoxybenzoic acid (80%, Aldrich, Milwaukee, WI), and HPLC solvents (Burdick and Jackson, Muskegon, MI) were used as received. HPLC was performed on a Waters instrument (Model 6000 pump, U6K injector, and either a Model R401 refractive index detector or Model 490 UV detector; Milford, MA). HPLC columns used were a Spherisorb ODS-II column (5 μm, C_{18}, 60 Å pore size, 4.6 × 250 mm; Custom LC, Houston, TX), a Vydac 201TP54 column (5 μm, C_{18}, 300 Å pore size, not end-capped, 4.6 × 250 mm; Separations Group, Hesperia, CA), and Dynamax 60A C_{18} (8 μm, 21.4 × 250 mm) and silica (8 μm, 10 × 250 mm) columns (Rainin, Woburn, MA). Conditions for gas-liquid chromatography-mass spectrometry (GLC-MS) are given in an earlier report (16) and in Table 1.

NMR spectra were acquired on an IBM AF300 spectrometer (300.1 MHz for ^1H and 75.5 MHz for ^{13}C). ^{13}C NMR spectra were acquired with magnetic homogeneity, acquisition time ($\geq$ 0.8 sec), digital resolution ($\sim$ 0.01 ppm), and line broadening (0–2 Hz) appropriate for good peak definition. Resolution enhancement was done by Gaussian multiplication with additional zero-filling to increase digital resolution. ^{13}C multiplicities were determined from DEPT experiments (17) employing either 90° or 135° read pulses. HETCOR spectra optimized for 125 Hz couplings were acquired using a DEPT pulse sequence with proton irradiation to eliminate non-geminal couplings (18). An observed ^1H chemical shift accuracy of $\sim$ 0.02 ppm over a range of δ 2.6–0.6 in the ^1H dimension was achieved by collecting $\sim$ 50 increments ($\sim$ 20 min of spectrometer time for a 20-mg sample). A smaller ^1H range and/or additional increments were used when better resolution was needed. For long-range HETCOR spectra (17), an INEPT pulse sequence was used with optimization for 10 Hz couplings and broadband decoupling in the ^1H dimension. The experiment was optimized for 10 Hz couplings by using a 50-msec defocusing delay (Δ_1) and a 30-msec refocusing delay (Δ_2). With 20-mg samples, most of the possible correlations between methyl protons and carbons 1–3 bonds away were visible after $\sim$ 2 hr (16 increments). An observed chemical shift accuracy of $\sim$ $\pm$ 0.05 ppm (δ 2.6–0.6 range) in the ^1H dimension permitted carbons coupled to 18-H_3 or 19-H_3 to be distinguished from carbons coupled to 30-H_3, 31-H_3, or 32-H_3.

A mixture of $\mathbf{II}_R$ and $\mathbf{II}_S$ was prepared from lanosterol, as described by Panini et al. (10), and the reaction products were separated by preparative reversed-phase HPLC (Dynamax 60A C_{18} column, eluted with methanol). A portion of this mixture (15 mg) was acetylated (pyridine and acetic anhydride), and the two epimers were separated by two passes through a Dynamax 60A silica column eluted with ethyl acetate–hexane 1:19 at 2 ml/min (retention times 30.2 and 32.4 min). The earlier-eluting epimer was 3β-acetoxy-24(S),25-epoxylanost-8-ene(6 mg): melting point(mp) 140–141.5°C (lit. mp 137–139°C, remelted at 143.5–144.5°C(19));MS, *m/z* (relative intensity) 484 (M^+, 1%), 469 (14%), 424 (4%), 409 (70%), 391 (15%), 355 (6%), 295 (2%), 109 (100%). The later-eluting epimer was 3β-acetoxy-24(R),25-epoxylanost-8-ene (7 mg): mp 194–195.5°C (lit. mp 194–197°C (19));MS, *m/z* (relative intensity) 484 (M^+, 3%), 469 (20%), 424 (5%), 409 (90%), 391 (20%), 355 (4%), 295 (3%), 109 (100%). A portion of the 24R epimer was saponified to the free sterol $\mathbf{II}_R$ by treatment with 10% KOH in 95% ethanol at 70°C for 1 hr.

A mixture of the acetates of $\mathbf{I}_R$ and $\mathbf{I}_S$ was synthesized from desmosterol acetate by analogy with a procedure (13) for the corresponding free sterols. The two epimers were separated and purified by normal phase HPLC, as described for the acetates of $\mathbf{II}_R$ and $\mathbf{II}_S$. This separation yielded 17.2 mg of the earlier-eluting component (35.8 min), 3β-acetoxy-24(S),25-epoxycholest-5-ene: mp 114.5–116.5°C;MS, *m/z* (relative intensity) 382 (M-AcOH, 44%), 367 (15%), 283 (16%), 255 (27%), 253 (63%), 213 (52%). The later-eluting component (18.0 mg, 38.4 min) was 3β-acetoxy-24(R),25-epoxycholest-5-ene: mp 110.5–112.5°C; MS, *m/z* (relative intensity) 382 (M-AcOH, 38%), 367 (13%), 283 (12%), 255 (24%), 253 (46%), 213 (51%). The overall yield of epoxidation to form the acetates of $\mathbf{I}_R$ and $\mathbf{I}_S$ was 62%. Saponification of a portion of the later-eluting acetate gave 24(R),25-epoxycholest-5-

en-3β-ol: mp 166–167°C (lit. mp 166.5–168°C (20), 164–165.5°C (5), 166–168.0°C (13)).

RESULTS

Epimeric mixtures of the epoxysterol acetates obtained by chemical syntheses were separated by preparative normal phase HPLC. **Fig. 1** illustrates the resolution and purity of the epimers on normal phase HPLC. The epimeric epoxylanosterol acetates were distinguished by their melting points (19), which differ by > 50°C. This identification was confirmed by saponifying the acetates to the free sterols and observing that the 24S epimer eluted before the 24R epimer on the Vydac column (10). The elution order of the acetates of $\mathbf{II}_R$ and $\mathbf{II}_S$ on normal phase HPLC (24-R epimer eluting last) was the same as that reported for the benzoates of $\mathbf{I}_R$ and $\mathbf{I}_S$ by normal phase HPLC (14) and for the acetates of $\mathbf{II}_R$ and $\mathbf{II}_S$ by preparative TLC (6). This elution order was also shown to be the case for the epoxycholesterol acetates by saponification of the later-eluting epimer to the free sterol, which was identified as the 24R epimer ($\mathbf{I}_R$) by its melting point and by its retention time on the Vydac column. The chromatographic retention times of the epoxysterol epimers are summarized in **Table 1**.

Epimeric mixtures of neither the epoxycholesterol acetates nor the epoxylanosterol acetates could be resolved by capillary GLC, and their mass spectra were very similar. This was consistent with the findings of others for the corresponding free sterols (6, 10, 13). ^{1}H NMR spectra of both pairs of epoxysterol acetate epimers were also virtually indistinguishable, as has been previously noted for the epoxylanosterol acetates (19). The ^{1}H NMR chemical shifts of resolved protons are listed in **Table 2**. The assignments were based on HETCOR experiments.

The ^{13}C NMR chemical shifts for each of the epoxysterol acetates are given in **Table 3**. The assignments for the epoxycholesterol acetates are based on reported assignments for desmosterol (21) after taking into account the effects of acetylation at C-3 (22) and of epoxidation of the side-chain double bond (23). The assignments of the side-chain carbons of the epoxylanosterol acetates were made by analogy with those of the epoxycholesterol ace-

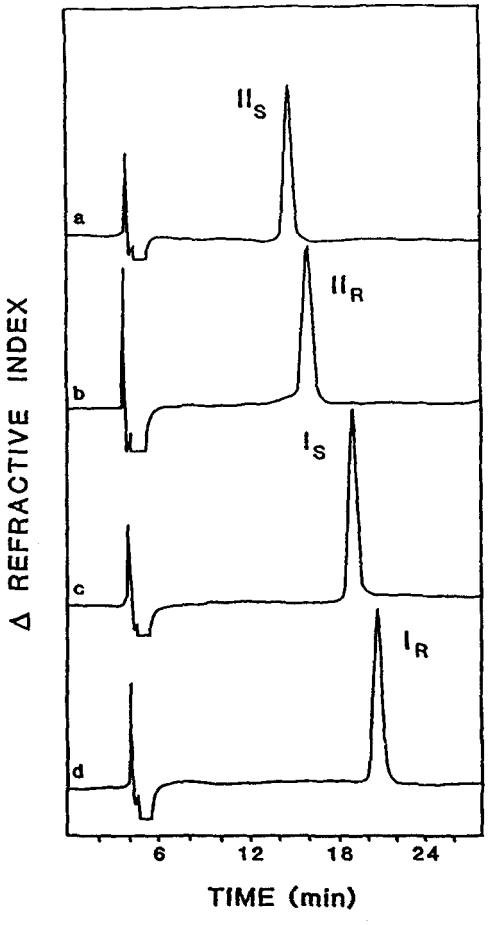

Fig. 1. Normal phase HPLC of the purified 24,25-epoxysterol acetates (Dynamax 60A column, 5% ethyl acetate in hexane at 4 ml/min): (a) and (b): 24R and 24S epimers of 24,25-epoxylanosterol acetate; (c) and (d): 24S and 24R epimers of 24,25-epoxycholesterol acetate. Refractive index detection (~ 200-250 μg of each sterol).

TABLE 1. Chromatographic retention times of 24,25-epoxysterols

Chromatography Conditions	Derivative	II_S	II_R	I_S	I_R
			min		
HPLC, Dynamax 60A, 10 × 250 mm EtOAc-hexane (1:19), 4 ml/min	acetate	15.4	16.8	19.5	20.9
HPLC, Spherisorb ODS-II, 4.6 × 250 mm MeOH-H_2O (98:2), 1.0 ml/min	acetate	18.2	18.2	17.0	17.0
HPLC, Vydac 201TP54, 4.6 × 250 mm MeOH, 0.8 ml/min	free sterol	13.0	14.2	11.2	12.4
GLC, DB-5 capillary, 0.25 mm × 15 m 6 psi He, 150–260°C at 10°C/min	acetate	7.99	7.98	7.22	7.21

tates. The ring carbons of the epoxylanosterol acetates were assigned by comparison with values for dihydrolanosterol (24) after correction for the C-3 acetylation shifts (25). Multiplicities derived from DEPT spectra required that C-6 and C-19 be inverted from the previous dihydrolanosterol assignments (24). The closely spaced signals for C-8 and C-9 and for C-12 and C-15 of the epoxylanosterol acetates were distinguished by their correlations to 32-H_3 and 19-H_3 in long-range HETCOR experiments. Assignments of other close pairs of signals (C-5, C-17; C-18, C-27; C-26, C-32) were confirmed based on their HETCOR spectra.

Small but significant differences (up to 0.3 ppm) in the ^{13}C NMR chemical shifts of the side chain and certain nearby carbons were observed for the acetates of the 24R and 24S epimers of 24,25-epoxycholesterol and 24,25-epoxylanosterol (Table 3). The chemical shifts of each epimer ($\mathbf{I_R}$, $\mathbf{I_S}$, $\mathbf{II_R}$, $\mathbf{II_S}$) were unchanged (± 0.02 ppm) whether measured as the individual epimer or as an epimeric mixture. We found the most reliable measure of the chemical shift differences between the 24R and 24S epimers to be the values determined from these spectra of epimeric mixtures, in which the temperature and solvent environment were identical for each epimer. These values are shown in **Fig. 2**. Although ^{13}C NMR chemical shifts are somewhat dependent on solvent and concentration, we found that the chemical shifts of the epoxysterols discussed here were reproducible to ± 0.02 ppm for 0.03–0.13 M concentrations of sterol in $CDCl_3$. Because the side-chain carbons (with the exception of C-26)[1] of the 24R and 24S epimers differ in chemical shift by magnitudes (0.09–0.30 ppm) considerably greater than the experimental error, samples of 24,25-epoxycholesterol or 24,25-epoxylanosterol can easily be identified as the 24R or 24S epimer from the data in Table 3. These ^{13}C NMR chemical shifts can be obtained in 1 hr from 1–2 mg of a single epimer or 2–4 mg of an epimeric mixture on a typical 300 MHz (for ^1H) spectrometer. Because acetylation

TABLE 2. ^1H NMR chemical shifts of 24,25-epoxysterol[a,b,c]

Hydrogen Atom	$\mathbf{I_R}$, $\mathbf{I_S}$ (acetates)		$\mathbf{II_R}$, $\mathbf{II_S}$ (acetates)	
3α-H	4.60	(m)	4.50	(dd)
18-H_3	0.684	(s)	0.694	(s)
19-H_3	1.018	(s)	1.003	(s)
21-H_3	0.941	(d)	0.916	(d)
24-H	2.69	(t)	2.69	(t)
26-H_3	1.310	(s)	1.310	(s)
27-H_3	1.266	(s)	1.267	(s)
30-H_3			0.882	(s)
31-H_3			0.882	(s)
32-H_3			0.877	(s)
Ac-H_3	2.03	(s)	2.05	(s)

[a] ^1H NMR data obtained at 300.1 MHz in 0.03–0.13 M $CDCl_3$ solution at ambient temperature (22°C). Referenced to internal $(CH_3)_4Si$ (0.0 ppm). Digital resolution ~0.001 ppm.

[b] Average of the chemical shifts of the 24R and 24S epimers. Chemical shifts of the individual epimers were within 0.001 ppm of these values. No doubling of methyl peaks was observed in mixtures of either pair of epimers except that strong resolution enhancement indicated 0.003 ppm splitting of the 26-H_3 resonance of $\mathbf{I_R}$/$\mathbf{I_S}$ and the 32-H_3 resonance of $\mathbf{II_R}$/$\mathbf{II_S}$.

[c] m, multiplet; dd, doublet of doublets (J = 4.7, 11.5 Hz); s, singlet; d, doublet (J = 6.0–6.5 Hz); t, triplet (J = 6.0 Hz). Resolution enhancement of the 32-H_3 resonance of a 1:1 mixture of $\mathbf{II_R}$ and $\mathbf{II_S}$ gave a 1:2:1 triplet, which apparently arose from long-range coupling (J = 1 Hz) and chemical shift differences (0.003 ppm) between $\mathbf{II_R}$ and $\mathbf{II_S}$.

TABLE 3. ^{13}C NMR chemical shifts of 24,25-epoxysterol acetates[a,b,c]

Carbon Atom	$\mathbf{I_R}$	$\mathbf{I_S}$	$\mathbf{II_R}$	$\mathbf{II_S}$
1	36.93	36.92	35.23	35.24
2	27.71	27.70	24.14	24.13
3	73.91	73.90	80.89	80.90
4	38.06	38.05	37.78	37.78
5	139.58	139.58	50.45	50.46
6	122.57	122.54	18.09	18.09
7	31.81	31.81	26.35	26.35
8	31.79	31.77	134.39	134.39
9	49.91	49.91	134.24	134.24
10	36.53	36.52	36.86	36.87
11	20.96	20.95	20.96	20.96
12	39.62	39.64	30.91	30.93
13	42.28	42.27	44.46	44.46
14	56.57	56.60	49.78	49.78
15	24.22	24.21	30.77	30.77
16	28.20	28.17	28.22	28.17
17	55.76	55.89	50.24	50.34
18	11.82	11.81	15.73	15.75
19	19.27	19.26	19.16	19.17
20	35.53	35.62	36.20	36.34
21	18.63	18.51	18.65	18.55
22	32.31	32.49	32.57	32.78
23	25.37	25.63	25.60	25.90
24	64.76	64.88	64.77	64.93
25	58.38	58.08	58.41	58.14
26	24.90	24.90	24.92	24.95
27	18.71	18.61	18.73	18.63
30			27.89	27.89
31			16.52	16.52
32			24.21	24.21
$\underline{C}OCH_3$	170.52	170.50	171.01	171.03
$C\underline{O}CH_3$	21.42	21.40	21.33	21.34

[a] ^{13}C NMR data measured at 75.5 MHz in $CDCl_3$ solution (0.4 ml, 0.03–0.13 M) at 22°C. Referenced to $CDCl_3$ at 77.0 ppm. Digital resolution ~0.01 ppm.

[b] NMR data from samples of the individual epimers.

[c] C-26 is defined as the *pro*-R methyl carbon, which is *anti* to C-24.

[1] The chemical shift differences between the 24R and 24S epimers of 24,25-epoxysterols can be ascribed to different population distributions of side-chain conformers for each epimer. Thus, the γ-gauche effects, which influence ^{13}C NMR chemical shifts, will generally be somewhat different for corresponding side-chain carbons of each epimer. Molecular mechanics calculations confirm that the population distribution of side-chain conformers is quite different for the two epimers. Because C-26 experiences no γ-gauche interactions and is remote from all nonbonded carbons in every conformation examined, the chemical shift of C-26 should be virtually unaffected by the stereochemistry of the 24,25-epoxy group.

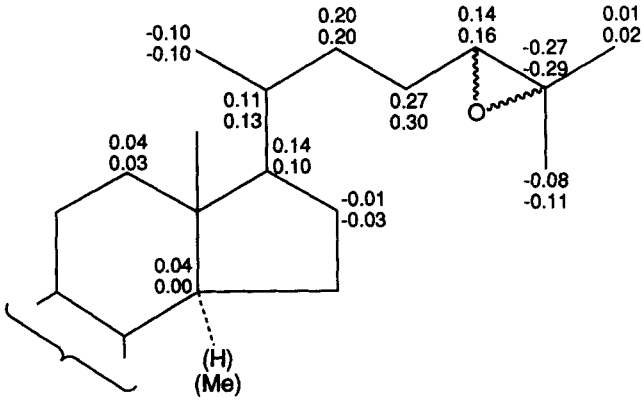

Fig. 2. ^{13}C NMR chemical shift differences (δ_S-δ_R) observed in 24RS mixtures of the acetates of 24,25-epoxycholesterol (upper values) and 24,25-epoxylanosterol (lower values). Differences of 0.01 ppm were also observed for C-5, C-8, C-9, C-11, and C-18 of 24,25-epoxylanosterol. Also, δ_S-δ_R was 0.01 ppm for C-6 and C-9 of 24,25-epoxycholesterol acetate.

and benzoylation shifts of 3β-hydroxy sterols are negligible for ring D and side-chain carbons, the chemical shift differences between the 24R and 24S epimers of epoxysterols can be expected to apply to the free sterols and benzoates as well.

DISCUSSION

The reported inhibitory effects of 24(S),25-epoxycholesterol on cholesterol biosynthesis (4) and HMG-CoA reductase activity (5, 6) combined with its detection as a naturally occurring sterol (5), have led to the suggestion that this oxysterol might represent a natural regulator of cholesterol synthesis in vivo (5). However, its importance in the regulation of HMG-CoA reductase has not been fully clarified (11, 12). Additional studies with I_S and other 24,25-epoxysterols are indicated. The HPLC and ^{13}C NMR procedures presented here provide valuable methodologies for the preparation and analysis of these epoxysterols.

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