Proton magnetic resonance identification and discrimination of stereoisomers of C_{27} steroids using lanthanide shift reagents

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Summary A simple proton magnetic resonance spectroscopic method is described for the identification and confirmation of several stereoisomeric pairs of C_{27} stanols as well as their keto and acetate derivatives related to cholesterol. The method, which involves the use of lanthanide shift reagents, is useful in distinguishing clearly between the isomeric pairs differing only in the geometry of a functional group and/or of the A/B-ring junction in the steroid skeleton.—**Iida, Takashi, M. Kikuchi, T. Tamura, and T. Matsumoto.** Proton magnetic resonance identification and discrimination of stereoisomers of C_{27} steroids using lanthanide shift reagents. *J. Lipid Res.* 1979. **20:** 279–284.

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The stereochemical isomers in a series of C_{27} stanols and stanones, in particular compounds related biosynthetically to cholesterol, frequently occur in lipid extracts of animal and plant organisms. For example, Eneroth, Hellström, and Ryhage (1) have reported the presence of cholesterol, cholestanol, coprostanol, and coprostanone metabolites in feces. Cholestanol has also been found as a major sterol in adrenal glands of the rat (2). In addition, Lederer et al. (3) have isolated epicoprostanol from ambergris.

The separation and identification of the stereoisomers of the C_{27} stanols differing only in the geometry of the C-3 functional group and/or of the A/B-ring junction in the steroid skeleton (e.g., cholestanol vs. epicholestanol and coprostanol vs. epicoprostanol) have been attempted by a variety of techniques, such as thin-layer chromatography (4, 5), gas–liquid chromatography (6, 7), and mass spectrometry (8, 9). However, no study has been made on adequate characterization by PMR spectroscopy of these compounds.

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We (10, 11) have previously reported on the effectiveness of $Eu(dpm)_3$, a LSR, in PMR spectroscopic studies of the structure and stereochemistry of many biologically important steroids and terpenoids. In the present paper, the method has been applied to the identification and discrimination of several stereoisomeric pairs of C_{27} stanols as well as their keto and acetate derivatives often found in lipid extracts. The use of $Eu(dpm)_3$ and $Eu(fod)_3$ as downfield reagents and $Pr(fod)_3$ as an upfield reagent has provided excellent results. To confirm the applicability of the present method, the spectra of several related sterols containing a double bond or additional methyl groups in the steroid nucleus were also investigated.

EXPERIMENTAL

Materials

Cholesterol was from the Riken Vitamin Oil Co., Tokyo, Japan. Coprostanol, epicoprostanol, and coprostanone were purchased from Gasukuro Works Ltd., Tokyo, Japan, and were used without further purification. Cholestanol and epicholestanol were prepared by catalytic hydrogenation of cholesterol and cholestanone, respectively, in the presence of PtO_2 catalyst. Cholestanone was prepared from cholestanol using sodium dichromate (12). 5α-Cholestan-7α-ol and 5α-cholestan-7β-ol were prepared from cholesterol by the method of Cremlyn and Shoppee (13) and purified by column chromatography on aluminum oxide. Δ^7-Cholestenol, $\Delta^{8(14)}$-cholestenol, and Δ^{14}-cholestenol were obtained from cholesterol according to the method of Cornforth, Gore, and Popjak (14). The preparations of 4α-methylcholestanol, 4,4-dimethylcholestanol, and 6α-methylcholstanol were previously described (15, 16). Acetylation of stanols was carried out using acetic anhydride and pyridine.

The LSR employed were $Eu(dpm)_3$ and deuterated $Eu(fod)_3$-d_{27} as downfield reagents and $Pr(fod)_3$ as an upfield reagent. The $Eu(dpm)_3$ and $Pr(fod)_3$ were

Abbreviations: PMR, proton magnetic resonance; LSR, lanthanide shift reagent(s); ISR, induced shift ratio; $Eu(dpm)_3$, tris-(dipivaloylmethanato)europium; $Eu(fod)_3$, tris(heptafluorobutanoylpivaloylmethanato)europium; $Pr(fod)_3$, tris(heptafluorobutanoylpivaloylmethanato)praseodymium. Trivial names and their corresponding systematic names are as follows: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; epicholestanol, 5α-cholestan-3α-ol; coprostanol, 5β-cholestan-3β-ol; epicoprostanol, 5β-cholestan-3α-ol; Δ^7-cholestenol, 5α-cholest-7-en-3β-ol; $\Delta^{8(14)}$-cholestenol, 5α-cholest-8(14)-en-3β-ol; Δ^{14}-cholestenol, 5α-cholest-14-en-3β-ol; 4α-methylcholestanol, 4α-methyl-5α-cholestan-3β-ol; 4,4-dimethylcholestanol, 4,4-dimethyl-5α-cholestan-3β-ol; 6α-methylcholestanol, 6α-methyl-5α-cholestan-3β-ol; cholestanone, 5α-cholestan-3-one; coprostanone, 5β-cholestan-3-one.

purchased from Dojin Laboratories, Kumamoto, Japan, and the deuterated Eu(fod)$_3$-d_{27} from E. Merck, Darmstadt, Germany. These reagents were stored under reduced pressure over P_2O_5 and used without further purification.

PMR spectra

All the PMR spectra were measured in $CDCl_3$ (0.4 ml) with tetramethylsilane (TMS) as an internal reference standard, at an ambient probe temperature of 34°C, using a Hitachi R-22 (90 MHz) spectrometer. After recording the normal PMR spectrum of a pure substrate, the lanthanide-induced shift spectra were obtained as follows. Exact amounts (2–4 mg) of a LSR were added in increasing amounts to the $CDCl_3$ solution of a known quantity of the substrate (ca. 10 mg) in the PMR sample tube, and spectra were recorded after each addition. Usually five or six such additions of the LSR were made for each sample. Chemical shift values were denoted as δ (ppm) relative to TMS.

RESULTS AND DISCUSSION

On successive additions of Eu(dpm)$_3$ (or Eu(fod)$_3$) shift reagent to substrate solutions, most methyl protons in compounds examined suffered the normal, expected shifts to downfield, except for particular protons mentioned below. The direction of the lanthanide-induced shifts was, however, reversed when Pr(fod)$_3$ was added to the same substrate solutions. Assignments for the methyl protons in the presence of the LSR were almost exclusively based on both the signal patterns observed (singlet or doublet) and the approximate distance measured between the coordinating site (oxygen-containing function) of a lanthanide metal ion and the protons.

With either LSR used, all the plots of the chemical shifts of the methyl protons vs. molar ratios of the LSR to the substrates gave straight lines within the molar ratios up to about 0.7. The line gradients (which are often called S values (17) and indicate the complexing ability of the substrate ligands with the lanthanide metal ion) for the corresponding protons usually decreased in the order of free alcohols > ketones > acetates, with the same concentration of the substrates: e.g., the observed values for the angular 18- and 19-methyl protons in various compounds (0.064 M) were as follows: (the Roman numeral refers to the compound in the respective table) cholestanol (I, 0.58, 2.71), cholestanone (XIX, 0.30, 1.55), and cholestanyl acetate (XIII, 0.07, 0.90). The decreasing order is in accord with the previous findings for triterpenoid derivatives (10). From the S values, the shifts induced by the LSR were then normalized in terms of the ISR values proposed by Wineburg and Swern (18) in the PMR spectra of the methyl ether derivatives of long-chain fatty acids. The results are listed in Tables 1–4; a minus sign represents an upfield shift of protons upon addition of the LSR.

Table 5 shows the S and ISR values observed for the methyl protons in cholesterol at various concentrations. It is apparent from the data that the S values for the same protons vary with the substrate concentrations, while the ISR values are more constant. The ISR values are therefore preferable for identification and analytical purposes.

As can be seen in **Table 1**, two stereoisomeric pairs of C-3 stanols, i.e., cholestanol (I) vs. epicholestanol (II), and coprostanol (III) vs. epicoprostanol (IV), have very similar spectral patterns of the methyl group regions in their normal spectra. However, each isomeric pair could easily be differentiated by successive addition of the LSR, because the ISR values for particular methyl protons were sufficiently different from one another based on the stereochemical configuration of the hydroxyl group in the epimers. Thus, in the epimer of the cholestane type (rings A/B, *trans*), the ISR value for the 18-methyl proton is larger in epicholestanol (II, *axial* 3α-OH) than in cholestanol (I, *equatorial* 3β-OH). Furthermore, the ISR values observed for each methyl proton in I were found to be very similar to those obtained in the corresponding unsaturated (V–VII) and methylated (VIII–X) sterols (rings A/B, *trans*; *equatorial* 3β-OH), suggesting that the presence or absence of unsaturation in B-, C-, or D-ring and of methyl substituents in the vicinity of the C-3 hydroxyl function in the steroid nucleus has no or little effect upon the magnitude of the values. In the epimer of the coprostane type (rings A/B, *cis*), on the other hand, the ISR value for the 18-methyl proton in coprostanol (III, *axial* 3β-OH) is smaller than that observed in epicoprostanol (IV, *equatorial* 3α-OH). A comparison of the compounds of the cholestane and coprostane types further revealed that the ISR values for the 18-methyl proton in the latter are larger than those in the former. Although such observations were found with either LSR examined, the differences in the ISR values between the epimeric pairs were somewhat influenced by the nature of the LSR; e.g., the use of the downfield shift reagents, Eu(dpm)$_3$ and Eu(fod)$_3$, allowed us to obtain a better discrimination between the epimer of coprostane type rather than that of the upfield shift reagent, Pr(fod)$_3$.

Epimeric 5α-cholestan-7-ols (rings A/B, *trans*) were also readily distinguished from one another, especially

TABLE 1. PMR data for methyl protons in C_{27} stanols and related sterols with or without the presence of LSR

Compounds	A/B-ring Junction	Configuration of OH Group	Methyl Groups[a]	Chemical Shifts[b]	ISR Values[c]		
					Eu(dpm)$_3$	Eu(fod)$_3$	Pr(fod)$_3$
I. Cholestanol	*trans*	*equatorial*	18-CH_3	0.65	0.20	0.21	−0.20
			19-CH_3	0.80	1.00	1.00	−1.00
			21-CH_3	0.89	0.09	0.08	−0.09
			26,27-*gem*-diCH_3	0.87	0.03	0.03	−0.03
II. Epicholestanol	*trans*	*axial*	18-CH_3	0.65	0.28	0.26	−0.32
			19-CH_3	0.77	1.00	1.00	−1.00
			21-CH_3	0.89	0.08	0.09	−0.08
			26,27-*gem*-diCH_3	0.86	0.02	0.01	−0.03
III. Coprostanol	*cis*	*axial*	18-CH_3	0.64	0.33	0.31	−0.34
			19-CH_3	0.94	1.00	1.00	−1.00
			21-CH_3	0.88	0.10	0.20	−0.19
			26,27-*gem*-diCH_3	0.85	0.06	0.07	−0.05
IV. Epicoprostanol	*cis*	*equatorial*	18-CH_3	0.64	0.46	0.54	−0.37
			19-CH_3	0.92	1.00	1.00	−1.00
			21-CH_3	0.89	0.23	0.25	−0.22
			26,27-*gem*-diCH_3	0.86	0.05	0.04	−0.09
V. Δ^7-Cholestenol	*trans*	*equatorial*	18-CH_3	0.53	0.23		
			19-CH_3	0.79	1.00		
			21-CH_3	0.90	0.09		
			26,27-*gem*-diCH_3	0.86	0.03		
VI. $\Delta^{8(14)}$-Cholestenol	*trans*	*equatorial*	18-CH_3	0.81	0.23		
			19-CH_3	0.69	1.00		
			21-CH_3	0.90	0.08		
			26,27-*gem*-diCH_3	0.86	0.03		
VII. Δ^{14}-Cholestenol	*trans*	*equatorial*	18-CH_3	0.89	0.23		
			19-CH_3	0.81	1.00		
			21-CH_3	0.90	0.09		
			26,27-*gem*-diCH_3	0.87	0.03		
VIII. 4α-Methylcholestanol	*trans*	*equatorial*	18-CH_3	0.65	0.20		
			19-CH_3	0.81	1.00		
			21-CH_3	0.90	0.07		
			26,27-*gem*-diCH_3	0.86	0.02		
			C-4α-CH_3[d]	0.94	2.46		
IX. 4,4-Dimethylcholestanol	*trans*	*equatorial*	18-CH_3	0.64	0.21		
			19-CH_3	0.85	1.00		
			21-CH_3	0.88[f]	0.08		
			26,27-*gem*-diCH_3	0.86	0.02		
			C-4α-CH_3[e]	0.95	2.57		
			C-4β-CH_3[e]	0.79	2.71		
X. 6α-Methylcholestanol	*trans*	*equatorial*	18-CH_3	0.65	0.23		
			19-CH_3	0.80	1.00		
			21-CH_3	0.91	0.09		
			26,27-*gem*-diCH_3	0.87	0.04		
			C-6α-CH_3[d]	0.85	0.34		
XI. 5α-Cholestan-7α-ol	*trans*	*axial*	18-CH_3	0.66	0.75	0.78	−0.79
			19-CH_3	0.78	1.00	1.00	−1.00
			21-CH_3	0.89	0.24	0.27	−0.25
			26,27-*gem*-diCH_3	0.86	−0.05	−0.05	0.05
					−0.06	−0.06	0.07
XII. 5α-Cholestan-7β-ol	*trans*	*equatorial*	18-CH_3	0.67	0.85	0.80	−0.79
			19-CH_3	0.77	1.00	1.00	−1.00
			21-CH_3	0.89	0.24	0.24	−0.27
			26,27-*gem*-diCH_3	0.86	−0.03	−0.03	0.01
							0.02

[a] The 18- and 19-methyl protons appear as a singlet, and 21-methyl and 26,27-*gem*-dimethyl protons as a doublet (J, 6.3–7.2 Hz).
[b] In δ (ppm) units relative to TMS measured at 90 MHz.
[c] The values are defined as the ratios of the S values for the 19-methyl proton to those for the other methyl protons; the minus sign represents an upfield shift of protons by successive additions of the LSR.
[d] Doublet (J, 7.2 Hz).
[e] Singlet.
[f] Estimated value due to signal overlapping.

TABLE 2. PMR data for methyl protons in C_{27} stanol acetates with or without the presence of LSR[a]

Compounds	Methyl Groups	Chemical Shifts	ISR Values		
			$Eu(dpm)_3$	$Eu(fod)_3$	$Pr(fod)_3$
XIII. Cholestanyl acetate	18-CH_3	0.64	0.07	0.10	−0.06
	19-CH_3	0.82	1.00	1.00	−1.00
	21-CH_3	0.89	−0.06	−0.08	0.09
	26,27-*gem*-diCH_3	0.86	−0.04	−0.06	0.07
XIV. Epicholestanyl acetate	18-CH_3	0.66	0.37	0.39	−0.30
	19-CH_3	0.80	1.00	1.00	−1.00
	21-CH_3	0.89	0.24	0.24	−0.24
	26,27-*gem*-diCH_3	0.86	0.11	0.13	−0.11
XV. Coprostanyl acetate	18-CH_3	0.65	0.24	0.20	−0.17
	19-CH_3	0.96	1.00	1.00	−1.00
	21-CH_3	0.90	−0.01	0.01	0.04
	26,27-*gem*-diCH_3	0.86	−0.05	−0.04	0.04
XVI. Epicoprostanyl acetate	18-CH_3	0.64	1.29	1.40	−1.21
	19-CH_3	0.93	1.00	1.00	−1.00
	21-CH_3	0.90[b]	0.59	0.80	−0.74
	26,27-*gem*-diCH_3	0.86	0.59	0.80	−0.76
XVII. 5α-Cholestan-7α-ylacetate	18-CH_3	0.64	0.95	0.89	−0.83
	19-CH_3	0.79	1.00	1.00	−1.00
	21-CH_3	0.90	0.54	0.54	−0.27
	26,27-*gem*-diCH_3	0.87	−0.01	−0.02	0.04
XVIII. 5α-Cholestan-7β-ylacetate	18-CH_3	0.67	1.20	1.03	−1.05
	19-CH_3	0.83	1.00	1.00	−1.00
	21-CH_3	0.89	0.39	0.29	−0.21
	26,27-*gem*-diCH_3	0.87	−0.10	−0.07	0.07

[a] See footnotes *a*–*c* in Table 1. The acetyl methyl proton occurs at 1.97–2.02 ppm in the absence of the LSR.
[b] Estimated value due to signal overlapping.

by adding $Eu(dpm)_3$; in the *equatorial* 7β-hydroxyl isomer (XII), the ISR value for the 18-methyl proton was larger than that observed in the *axial* 7α-hydroxyl one (XI). In these compounds, however, more notable was the unexpected shift upfield with $Eu(dpm)_3$ and $Eu(fod)_3$ [or downfield with $Pr(fod)_3$] of the 26,27-*gem*-dimethyl protons at the C-17 side chain (discussed below in detail). In addition to the opposite-direction shift, it is also of considerable importance to note the appearance of two pairs of doublets for the protons that were rendered nonequivalent by progressive addition of certain LSR, e.g., $Pr(fod)_3$. This signal splitting appears to be related to the restricted free rotation of the C-25 terminal isopropyl moiety, probably due to its spatial proximity to the coordinating LSR.

Conversion of the stanols into their acetate derivatives provided further confirmatory evidence for the structure of these compounds (**Table 2**). Thus, compared with the measurement of the free alcohols, the differences in the ISR values for each methyl proton between epimeric pairs were found to be much more

TABLE 3. PMR data for protons attached to hydroxyl- or acetoxyl-substituted carbon atom with or without the presence of $Eu(dpm)_3$ shift reagent

Compounds	Position and Configuration of protons	Chemical Shifts	Half-height Width $W_{1/2}$	ISR Values[b]
				$Eu(dpm)_3$
			Hz	
I. (XIII)[a]	*axial* 3α-H	3.55 (4.64)	24.1 (18.6)	3.5 (5.1)
II. (XIV)	*equatorial* 3β-H	4.00 (4.98)	9.8 (8.0)	4.4 (11.3)
III. (XV)	*equatorial* 3α-H	4.06 (5.04)	7.9 (8.1)	6.0 (9.3)
IV. (XVI)	*axial* 3β-H	3.59 (4.68)	24.5 (22.6)	8.6 (49.4)
XI. (XVII)	*equatorial* 7β-H	3.78 (4.84)	6.2 (10.6)	5.5 (10.2)
XII. (XVIII)	*axial* 7α-H	3.32 (4.50)	21.3 (17.2)	5.1 (9.9)

[a] The data for acetate derivatives (XIII–XVIII) are designated in parentheses.
[b] See footnote *c* in Table 1.

TABLE 4. PMR data for methyl protons in C_{27}-stanones with or without the presence of LSR[a]

Compounds	A/B-ring Junction	Methyl Groups	Chemical Shifts	ISR Values		
				$Eu(dpm)_3$	$Eu(fod)_3$	$Pr(fod)_3$
XIX. Cholestanone	*trans*	18-CH_3	0.68	0.19	0.26	−0.31
		19-CH_3	1.00	1.00	1.00	−1.00
		21-CH_3	0.90	0.08	0.11	−0.07
		26,27-*gem*-diCH_3	0.86	0.01	0.02	−0.02
XX. Coprostanone	*cis*	18-CH_3	0.69	0.35	0.35	−0.40
		19-CH_3	1.01	1.00	1.00	−1.00
		21-CH_3	0.91	0.18	0.19	−0.22
		26,27-*gem*-diCH_3	0.87	0.06	0.05	−0.01

[a] See footnotes *a–c* in Table 1.

prominent in their acetate derivatives, though the magnitude of the S values for the methyl protons was relatively small as noted above. It is noticed here that, in epicoprostanyl acetate (XVI), the 18-methyl proton moved somewhat more rapidly than did the 19-methyl proton on successive additions of the LSR. Of further interest was the unexpected direction shift observed for the C-17 side chain signals (doublets for the 21-methyl and 26,27-*gem*-dimethyl protons) in cholestanyl acetate (XIII) and coprostanyl acetate (XV), in which the C-3 functional group had a β-configuration. These signals showed an upfield shift with $Eu(dpm)_3$ [or $Eu(fod)_3$] and a negative ISR, while the normal, expected shift downfield was observed for the other signals including the C-3 acetyl methyl proton with the same LSR; reverse results were obtained with $Pr(fod)_3$. A similar observation was also found in the lanthanide-induced shift spectra of the 26,27-*gem*-dimethyl protons of epimeric 5α-cholestan-7-ylacetates (XVII and XVIII) as well as their free alcohols (XI and XII).

The above phenomena in the acetate derivatives are presumably attributable to a particular geometrical disposition of the coordinating lanthanide metal ion against the methyl protons under consideration. According to previous reports (19, 20), the direction of the lanthanide-induced shifts depends on the magnitude of θ_i values of the angle dependence term $(3\cos^2\theta_i - 1)$ in the pseudo-contact shift equation, where θ_i is the internuclear angle between the vector connecting the metal atom to the nucleus i and the principal magnetic axis of the molecular complex. In the equation, when θ_i has a value from 54.5 to 125°, the angle dependence term becomes negative and an opposite-direction shift is observed. Hence, the opposite direction shift observed for the 21-methyl and 26,27-*gem*-dimethyl protons in some compounds (XI, XII, XIII, XV, XVII, and XVIII) must fall into the category with the θ_i values estimated to be in the range.

Next, we examined the effect of LSR on protons attached to the same carbon atoms as a hydroxyl or acetoxyl function. Such protons are in the immediate vicinity of the complexing site and would be strongly affected by the LSR. In fact, the 3α- and 3β-protons (or 7α- and 7β-protons) in stanols (I–XII) and their acetate derivatives (XII–XVIII) showed very large shifts in the expected direction on successive additions of the LSR (**Table 3**). The magnitude of the ISR values for these protons was dependent on their α- or β-stereochemical configuration. In the case of C-3 stanols, the ISR values were appreciably larger for the 3β-proton (II and IV) than for the corresponding 3α-proton (I and III). The differences in the ISR values between the epimeric C-3 stanols became much more pronounced in the C-3 acetate derivatives, similar to those found for methyl protons. More-

TABLE 5. S and ISR values observed for methyl protons at various concentrations of cholesterol in the presence of $Eu(dpm)_3$ shift reagent

Conc. of substrate ($\times 10^{-1}$ M)	S Values[a]				ISR Values			
	18-CH_3	19-CH_3	21-CH_3	26,27-*gem*-diCH_3	18-CH_3	19-CH_3	21-CH_3	26,27-*gem*-diCH_3
0.65	0.72	3.15	0.31	0.10	0.23	1.00	0.10	0.03
1.30	0.81	3.40	0.33	0.15	0.24	1.00	0.10	0.04
1.94	0.88	3.55	0.35	0.19	0.25	1.00	0.10	0.05
2.57	0.93	3.76	0.39	0.20	0.25	1.00	0.10	0.05

[a] The values show the gradients of the straight lines obtained from the plots of the chemical shifts of the methyl protons vs. molar ratios of $Eu(dpm)_3$/cholesterol.

over, the position and shape (half-height width W½) of the proton resonances in the normal spectra were influenced by their *axial* or *equatorial* nature, in agreement with those reported in the literature (21). Therefore, the above characteristics for such protons are also of importance in establishing the epimeric configuration of an oxygen-containing function, particularly at C-3 position, in the steroid nucleus, though the signal intensity is relatively weak.

The normal spectra of a stereoisomeric pair of cholestanone (XIX, rings A/B, *trans*) and coprostanone (XX, rings A/B, *cis*) are virtually identical, as indicated in **Table 4**. The two isomers, however, were readily distinguishable from one another; the ISR values for the 18- and 21-methyl protons were appreciably larger in XX.

Our present method of PMR spectroscopy, in conjunction with LSR, proved to be a simple and efficient method for confirming the A/B-ring junction and/or the conformation of a functional group in steroids and, therefore, may be applied to the structural elucidation of many closely related sterols isolated from natural sources or from in vitro metabolic studies.

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