Synthesis and structure of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol isolated from the urine and feces of a patient with sitosterolemia and xanthomatosis[1]

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Abstract The urine and feces of a patient with the rare inherited lipid storage disease, sitosterolemia and xanthomatosis, were analyzed. Substantial quantities of C_{26}-bile alcohol, 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol along with 5β-cholestane-3α,7α,12α,24-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24R,25-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol were found. The structure of the C_{26}-bile alcohol was confirmed by direct comparison (gas–liquid chromatography–mass spectrometry and thin-layer chromatography) with a standard sample synthesized from cholic acid. The configurational assignment at C-24 was determined by lanthanide-induced circular dichroism Cotton effect measurements. The increased excretion of these C_{26}- and C_{27}-bile alcohols suggests an abnormality of bile acid biosynthesis in this disease. —**Dayal, B., G. S. Tint, V. Toome, A. K. Batta, S. Shefer, and G. Salen.** Synthesis and structure of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol isolated from the urine and feces of a patient with sitosterolemia and xanthomatosis. *J. Lipid Res.* 1985. **26:** 298–305.

Supplementary key words bile alcohols

The association of tendon xanthomatosis and normal cholesterol levels has been described in two sterol storage diseases, cerebrotendinous xanthomatosis (CTX) in which large amounts of cholestanol are present in plasma and tissues, and sitosterolemia with xanthomatosis in which the plant sterols sitosterol, campesterol (24ξ-methyl cholesterol),[3] and stigmasterol are detected in substantial quantities in plasma and xanthomas (1–8). Recently, we have studied the plasma and fecal sterols of four patients with the rare lipidosis, sitosterolemia with xanthomatosis, who exhibited tendon xanthomas with elevated plasma levels of sitosterol and campesterol but normal plasma cholesterol concentrations (9–11).

In the present study on one patient with the same syndrome, we have now identified substantial amounts of polyhydroxylated neutral sterols in urine and feces. Thin-layer chromatography combined with gas–liquid chromatography–mass spectrometry (GLC–MS) indicated the presence of bile alcohols, namely 5β-cholestane-3α,7α,12α,24-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24R,25-pentol, 5β-cholestane-3α,7α,12α,25,26-pentol, and a C_{26}-bile alcohol which was tentatively identified as 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25-pentol (12). While 5β-cholestane tetrols and 5β-cholestane pentols are also found in CTX and patients with cholestasis, the C_{26}-bile alcohol has not been described in that disease (13–23).

Recently, Karlaganis et al. (24, 25) and Ludwig et al. (26) have identified the glucuronide of this C_{26}-bile alcohol as the major component in the urine of healthy subjects and patients with liver disease. However, the chirality at C-24 and C-25 has not been determined. The present study was performed in order to further elucidate the structure of this compound and establish the stereochemistry at C-24 by circular dichroism spectroscopy.

Abbreviations: Systematic names of steroids referred to in the text by their trivial names are: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; campesterol, 24ξ-methylcholesterol; stigmasterol, 24α-ethylcholest-5,22E-dien-3β-ol; coprostanol, 5β-cholestan-3β-ol; 3-epicoprostanol, 5β-cholestan-3α-ol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid. CTX, cerebrotendinous xanthomatosis; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; I.R., infra red; TMSi, trimethylsilyl; RRT, relative retention time; THF, tetrahydrofuran; MS, mass spectrometry; CD, circular dichroism.

[1]Dayal, B., G. S. Tint, and G. Salen. "Structure and stereochemistry of a new C_{26}-sterol excreted by patients with neutral sterolemia and xanthomatosis (NSX)." Presented in part at the National Meeting of the American Chemical Society held in Atlanta, Georgia, March, 1981.

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[3]Since the term "campesterol" is stereospecific and the GLC does not distinguish it from its co-occurring epimer, 22-dihydrobrassicasterol, campesterol has been replaced with "24ξ-methyl cholesterol."

EXPERIMENTAL PROCEDURES

Physical measurements

Melting points were determined on a Thermolyne apparatus (Thermolyne Corp., Dubuque, IA), model MP-12600, and are uncorrected.

Infrared spectra were recorded on a Perkin-Elmer (Norwalk, CT) model 421 grating spectrophotometer in Nujol. Absorption frequencies are quoted in reciprocal centimeters.

GLC

The bile alcohols, as the TMSi-derivatives, were analyzed on a 180 cm × 4 mm column packed with 3% OV-17 on 80/100 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA); column temp. 230°C (Hewlett-Packard model 7610 gas chromatograph, Palo Alto, CA).

Mass spectra of the bile alcohols were obtained with a Varian MAT-111 gas chromatograph–mass spectrometer as described previously (27).

TLC

The bile alcohols were separated on silica gel G plates (Brinkmann, Westbury, NJ, 0.25-mm thickness) and the spots were visualized either with iodine or phosphomolybdic acid (3.5% in isopropanol).

Optical measurements

The CD measurements were carried out on a Jasco J-20 instrument at 24°C, under a stream of high purity dry N_2, with a cell thickness of 0.1 cm. The coefficient of dichroic absorption, $\Delta\epsilon$, was calculated from the molar ellipticity (θ) by the following equation: molar ellipticity (θ) = $3300\Delta\epsilon$ (28). Both the molar ellipticity (θ) and $\Delta\epsilon$ are expressed in degree × cm^2 × $dmol^{-1}$. The Cotton effect was measured at its maximum value, around 310 mm, and was found to correlate with the chirality of the two hydroxy groups (18, 19, 28, 29). For actual measurement, a 1:1 mixture of the bile alcohol and $Eu(fod)_3$ was made in dry chloroform (ethanol-free) so that the concentration of the solutes was 2×10^{-4} M. The CD was then measured after 30–60 min.

Clinical

Feces and urine were obtained from a subject (aged 17, now deceased) with sitosterolemia and xanthomatosis. This patient had developed tendon and tuberous xanthomas associated with elevated plasma plant sterol concentrations [sitosterol 13 ± 5 mg/dl, (normal <1.0); 24ξ-methylcholesterol 7.1 ± 2.5 mg/dl, (normal <1.0)] characteristic of this syndrome (1–11).

Sterol determinations

Gas–liquid chromatography was employed for the quantitative determinations of plasma and fecal sterols and 5α-stanols as the TMSi-ether derivatives (11, 13). This method separated cholesterol from the higher molecular weight plant sterols and bile alcohols which are recorded in Tables 1, 2 and **Fig. 1**.

Prior to GLC, 5α-stanols were separated from the unsaturated sterols by argentation thin-layer chromatography utilizing the solvent system chloroform–acetone 97:3 (v/v). Plates were visualized with Rhodamine B, and areas corresponding to unsaturated and saturated sterol standards run on the same plate were scraped, extracted with diethyl ether, and analyzed by GLC–MS.

Determination of urinary bile alcohols

Dried methanol eluates from the Sep-pak extraction (30) of unhydrolyzed urine (2 to 10 ml, depending on the expected concentration of bile alcohols) were dissolved in acetate buffer (5 mol/l, pH 4.8) and *Helix pomatia* enzyme

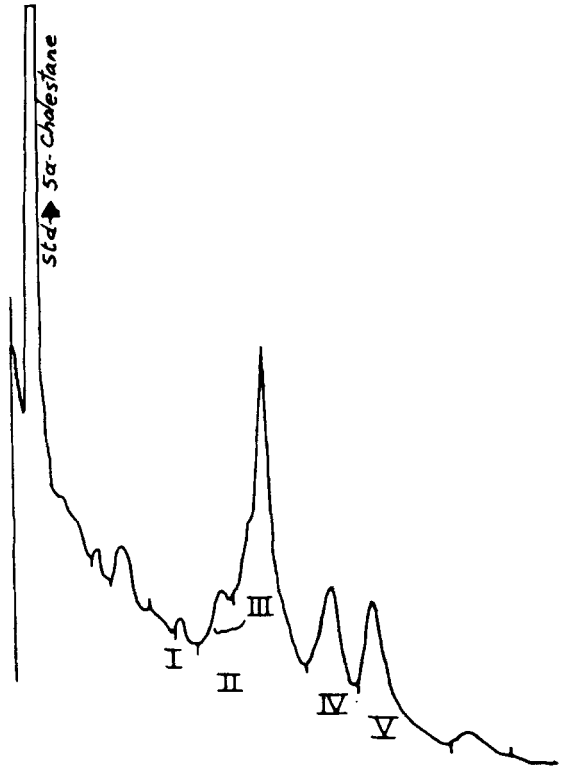

Fig. 1. GLC chromatogram of the TMSi ether derivatives of bile alcohols isolated from the feces of a patient with sitosterolemia and xanthomatosis. Column: 3% OV-17 at 270°C and N_2 flow 40 ml/min. O, 5α-Cholestane; I, 5β-cholestane-3α,7α,12α,24ξ-tetrol; II, 5β-cholestane-3α,7α,12α,25-tetrol; III, 26 (or 27)-nor-5β-cholestane-3α,7α,12α,-24S,25ξ-pentol; IV, 5β-cholestane-3α,7α,12α,24R,25-pentol; V, 5β-cholestane-3α,7α,12α,25,26-pentol.

preparation was added (Sigma type I, sulphatase and β-glucuronidase). Hydrolysis was allowed to proceed for 48 hr at 37°C and the liberated steroids were applied onto a Sep-pak C_{18} cartridge, which was previously washed with 10 ml of methanol and 10 ml of distilled water, respectively. After washing three times with 5 ml of distilled water, the cartridge was eluted with two portions of 2 ml of methanol. The eluate was evaporated to dryness at 60°C under N_2. Then trimethylsilylation was performed as described previously (27). Quantitation (by GLC) was done by comparison to peak heights relative to that of the internal standard.

Preparation of reference compounds

5β-Cholestane-3α,7α,12α,24α-tetrol (24R) (mp 180–182°C), 5β-cholestane-3α,7α,12α,24β-tetrol (24S) (mp 181–183°C), 5β-cholestane-3α,7α,12α,25-tetrol (mp 188.5–190.5°C), 5β-cholestane-3α,7α,12α,24R,25-pentol (mp 199–201°C), and 5β-cholestane-3α,7α,12α,25,26-pentol (mp 171–173°C) were synthesized according to Dayal et al. (16, 31, 32). 26 (or 27)-Nor-5β-cholestane-3α,7α,12α,-24S,25ξ-pentol (mp 194–200°C) was synthesized as described below.

Synthesis of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol

*Preparation of 3α,7α,12α-triformyloxy-24-oxo-26 (or 27)-nor-5β-cholestane (**Fig. 2,** III).* 3α,7α,12α-Triformyloxycholic acid chloride [prepared from 0.9 gm of 3α,7α,12α-triformyloxycholic acid (II, mp 205–207°C) and 2 ml of oxalyl chloride (31)] was dissolved in 5 ml of anhydrous benzene and poured over 50 ml of a cold ethereal solution of diazoethane [prepared from 5 g of nitrosoethylurea (33)]. The reaction mixture was left overnight at 0°C and the solvent was evaporated under a current of dry N_2. The yellow oily product obtained showed a major spot, on TLC, R_f = 0.6 [silica gel G plate, solvent system, benzene–ethyl acetate 95:5 (v/v)] in addition to a minor spot, R_f = 0.8. The faster moving compound had the same R_f value as ethyl-3α,7α,12α-triformyloxycholate but could not be removed by the conventional methods used for purification.

Preparation of 25-acetoxy-3α,7α,12α-triformyloxy-26 (or 27)-nor-5β-cholestan-24-one (Fig. 2, IV). To a cold solution of the diazoketone (III, 0.122 mmol) in 8 ml of dry methylene chloride was added dropwise with stirring 1.4 ml of concentrated hydrochloric acid. Stirring was continued for an additional 1 min at 0°C, and the reaction mixture was

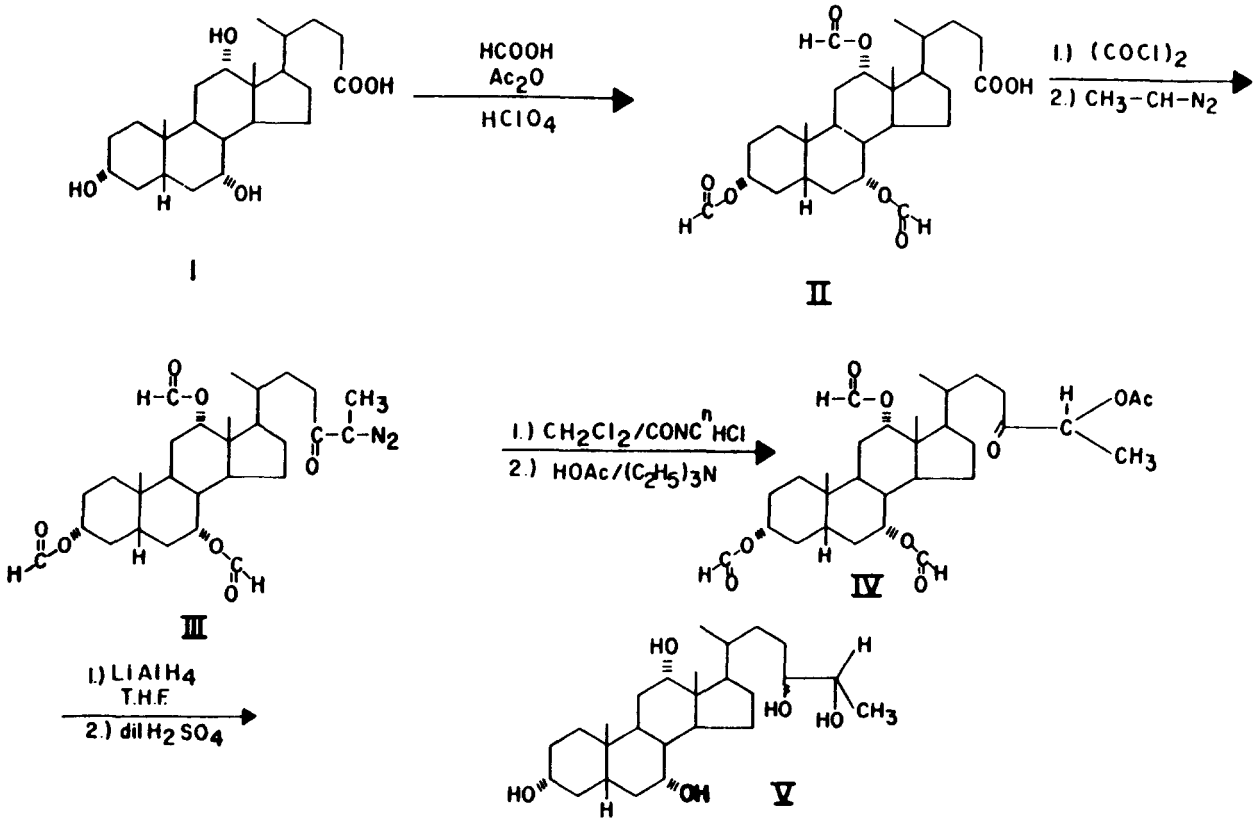

Fig. 2. Synthesis of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol. I, Cholic acid; II, 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid; III, 3α,7α,12α-triformyloxy-25-diazo-24-oxo-26 (or 27)-nor-5β-cholestane; IV, 25-acetoxy-3α,7α,12α-triformyloxy-26 (or 27)-nor-5β-cholestan-24-one; V, 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24ξ,25-pentol.

allowed to reach room temperature (during this procedure evolution of nitrogen was observed). Water (10 ml) was added, the organic phase was washed to neutrality and evaporated to yield the crude 25-chloro-3α,7α,12α-triformyloxy-26 (or 27)-nor-5β-cholestan-24-one (IV) as a yellow foam. To a solution of this product in 20 ml of acetone was added 4 ml of glacial acetic acid and 5 ml of dry triethylamine. The yellow solution was refluxed for 4 hr, poured into 100 ml of methylene chloride, washed with 10% potassium carbonate solution (3 × 20 ml) and with water (2 × 20 ml). The solution was dried over anhydrous Na_2SO_4 and evaporated to give 65 mg of the crude 25-acetoxy-3α,7α,12α-triformyloxy-26 (or 27)-nor-5β-cholestan-24-one as a yellowish solid.

Preparation of 26 (or 27)-nor-5β-cholestan-3α,7α,12α,24S,25-pentol (Fig. 2, V). To a solution of compound IV (65 mg) in 20 ml of THF (freshly distilled from $LiAlH_4$) was added with stirring $LiAlH_4$ (45 mg) in one portion. The reaction mixture was refluxed for 3 hr. After cooling, ethyl acetate (6 ml) was added carefully to destroy excess $LiAlH_4$, followed by a dilute solution of H_2SO_4. The product was extracted with ethyl acetate (2 × 25 ml), the ethyl acetate layer was washed with 5% $NaHCO_3$ solution and then with water to neutrality. Evaporation of the solvent yielded 43 mg (66%) of a crude product (Fig. 2, V) which on crystallization from ethyl acetate–methanol yielded 28 mg of colorless crystals, mp 194–200°C; GLC retention time of the TMSi ether: 3.04 (relative to 5α-cholestane RT, 5.11 min.); I.R. (Nujol), 344 cm^{-1} (OH). Upon thin-layer chromatography [solvent system, chloroform–acetone–methanol 70:50:20 (v/v/v)] the crystalline material yielded two bands, R_f 0.44 (A) and 0.48 (B) which were isolated by preparative TLC and characterized by mass spectrometry (**Fig. 3**).

Isolation of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol from feces of a patient with sitosterolemia and xanthomatosis

Feces were lyophilized and the neutral sterols and bile alcohols were isolated by a combination of column chromatography on neutral alumina and TLC [solvent system, chloroform–acetone–methanol 70:50:20 (v/v/v)] as described previously (12, 16). The compounds with the following R_f values were identified by direct comparison (GLC–MS) with authentic standards: 5β-cholestane-3α,7α,12α,24 (R)-tetrol, 0.60; 5β-cholestane-3α,7α,12α,24β-(S)-tetrol, 0.64; 5β-cholestane-3α,7α,12α,25-tetrol, 0.61; 5β-cholestane-3α,7α,12α,24R,25-pentol, 0.48; 5β-cholestane-3α,7α,12α,25,26-pentol, 0.47 (16, 17, 31, 32). In addition, a band corresponding to R_f 0.44 was isolated and characterized as 26 (or 27)-nor-5β-cholestane-3α,7α,12α,-24S,25ξ-pentol by comparison (TLC and GLC–MS) with the synthetic sample (see above). The CD spectrum of the compound in the presence of Eu(fod)$_3$ showed $\Delta\epsilon_{310}$

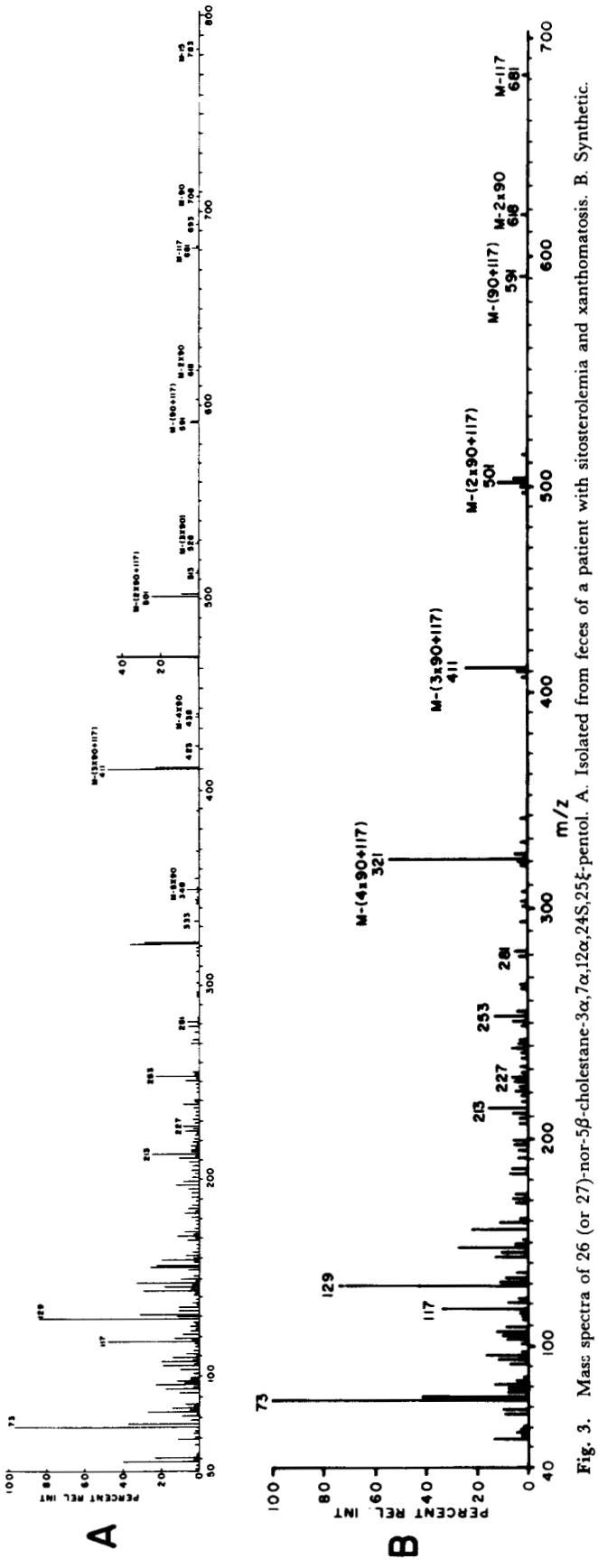

Fig. 3. Mass spectra of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol. A. Isolated from feces of a patient with sitosterolemia and xanthomatosis. B. Synthetic.

= −1.97 degree × cm^2 × dmol^{-1} (first Cotton effect), and $\Delta\epsilon_{285}$ = +1.67 degree × cm^2 × dmol^{-1} (second Cotton effect) (see Table 3 and **Fig. 4**).

Periodate oxidation of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol

26 (or 27)-Nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol (1 mg) was oxidized with sodium metaperiodate (2 mg) in 0.3 ml of water and the product was isolated and further oxidized with 1 drop of 8 N chromic acid in 0.5 ml of acetone according to Karlanganis et al. (24).

RESULTS AND DISCUSSION

The present investigation describes studies performed on a patient who had tendon and tuberous xanthomas and normal plasma cholesterol levels. No other clinical abnormalities were noted. Plasma levels of the plant sterols sitosterol and 24ξ-methyl cholesterol were found to be in high concentrations and other sterols such as cholestanol, sitostanol, and 24ξ-methyl campestanol were also present in large amounts (9, 11). **Table 1** illustrates the neutral sterols present in the feces of this patient. Coprostanol and its 24-ethyl derivative constituted the major part of the neutral sterols. The accumulation of plant sterols in this disease was attributed to their greatly increased intestinal absorption (5). Substantial amounts of polyhydroxy sterols, 5β-cholestane-3α,7α,12α,24ξ-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24R,25-pentol, 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24S,25ξ-pentol, and 5β-cholestane-3α,7α,12α,25,26-pentol, were found in the fecal extract (Fig. 1, **Table 2**). Examination of the urine also revealed a similar bile alcohol pattern. While the relative proportion of these bile alcohols varied considerably, two compounds (listed in order of abundance), namely 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24,25-pentol (96 μg/100 ml) and 5β-cholestane-3α,7α,12α,25,26-pentol (90 μg/100 ml), constituted the major part of the bile alcohols in urine.

TABLE 1. Neutral sterols identified in the feces of a patient with sitosterolemia and xanthomatosis[a]

Sterols	RRT[b] (3% OV-17 column)	% of Total Fecal Sterols
Cholesterol	1.91	8.6
3-Epicoprostanol	1.65	0.2
Coprostanol	1.40	23.2
24-Ethyl coprostanol	2.17	56.7
Sitosterol	2.90	6.5
Campesterol	2.37	5.3

[a]Extracted from 4 g of lyophilized fecal specimen (see Experimental Procedures).
[b]The neutral sterols were injected as their TMSi ether derivatives. The values in the Table are relative to 5α-cholestane (RT, 5.11 min).

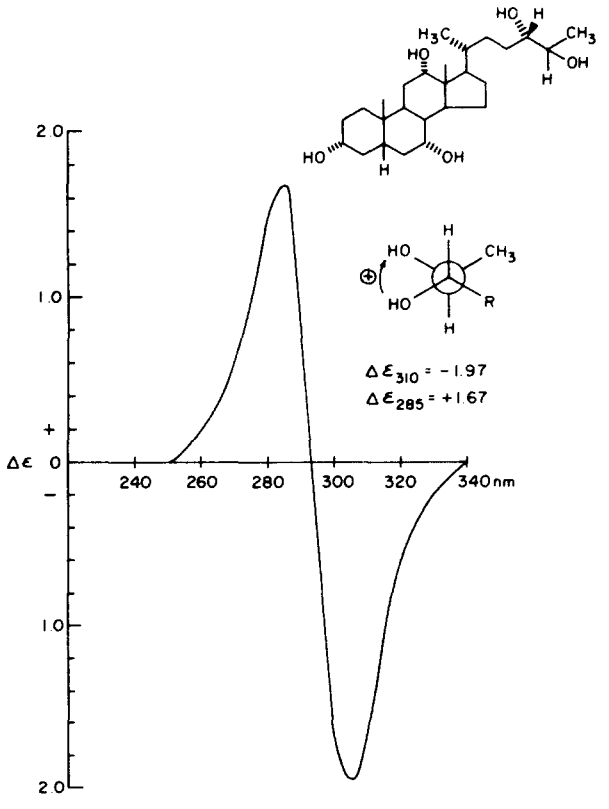

Fig. 4. Circular dichroism of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,-24S,25ξ-pentol (2 × 10^{-4} M) after addition of Eu(fod)$_3$ (2 × 10^{-4} M).

While 5β-cholestanetetrols and -pentols of the types described in CTX and cholestatic patients were found (13–23), the structure of the C_{26}-bile alcohol was tentatively identified as 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24,25-pentol from a study of the mass spectrum of its TMSi ether and periodate oxidation. The molecular ion peak was not observed but the M-15 peak 0.1% was seen at m/z 783 (Fig. 3A). A series of peaks formed by loss of 117 mass units and consecutive losses of trimethyls silanol were observed at m/z 681, 591, 501, 411, and 321, respectively. The peak at m/z 117 was due to a $CH_3 \cdot CH \cdot OTMSi$ group in the side chain. The peak at m/z 253 was due to the loss of side chain from the fragment at m/z 321. A vicinal glycol system in the side chain was confirmed by periodate oxidation of the compound followed by chromic acid oxidation when 3,7,12-triketo-5β-cholanoic acid was obtained. A 27-nor-pentol with this structure has recently been reported by Karlaganis et al. (24, 25) and Ludwig et al. (26), and the mass spectrum of the TMSi ether of our compound (Fig. 3A) closely resembled that reported for the TMSi ether of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,-24S,25ξ-pentol.

In order to further confirm the structure of this compound, it was synthesized from cholic acid via the diazo-

TABLE 2. Bile alcohols identified in the feces of a patient with sitosterolemia and xanthomatosis[a]

Bile Alcohol	RRT[b] (3% OV-17 column)	Feces	% of Total Bile Alcohol
		mg/g	
5β-Cholestane-3α,7α,12α,24(α and β)-tetrols[c]	2.30	0.009	1.15
5β-Cholestane-3α,7α,12α,25-tetrol	2.66	0.08	6.94
26 (or 27)-Nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol	3.04	0.61	55.16
5β-Cholestane-3α,7α,12α,24R,25-pentol[c]	3.65	0.21	19.33
5β-Cholestane-3α,7α,12α,25,26-pentol	4.04	0.19	17.42
Total mg/g of feces		1.099	100.00

[a]Extracted from 4 g of fecal specimens (See Results section).
[b]Relative retention time to 5α-cholestane, 5.11 min.
[c]The configuration at C-24 was established by TLC (16, 17, 20, 31, 32).

ketone III (Fig. 2) and two compounds were obtained. The compound with the lower R_f value was identical with the biosynthetic material on TLC and GLC, and the mass spectra of the two compounds were identical (Fig. 3B). The compound with the higher R_f value also showed the same GLC–MS behavior and was probably an isomer (at C-24 or C-25). The chirality at C-24 in the biosynthetic compound was established from the lanthanide-induced circular dichroism Cotton effect measurements. Dillon and Nakanishi (34) have shown that for secondary-tertiary acyclic compounds the sign of the first Cotton effect coincides with the glycol chirality (see compounds 1 and 2, **Table 3**), whereas for acyclic primary-secondary and secondary-secondary compounds, the sign is opposite to the glycol chirality of the conformer having the large groups to the rear. Based on this empirical model, the chirality at C-24 in the 26 (or 27)-nor-pentol (compound 3, Table 3 and Fig. 4) was assigned as 24S.

There are only three previous reports regarding the occurrence of C_{26} sterols in humans. Ikekawa et al. (35) identified 22-*trans*-27-nor-cholesta-5,22-dien-3β-ol from the urine of a girl with congenital adrenal hyperplasia. Kibe et al. (23) reported the presence of 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol in the bile from a patient with cholestasis due to gallstones in the common bile duct. Karlaganis and co-workers (24, 25) have recently reported the presence of a considerable amount of the glucuronide of 26 (or 27)-nor-5β-cholestane-3α,7α,12α,-24ξ,25ξ-pentol in the urine of a child with neonatal cholestasis, while Ludwig et al. (26) also found the glucuronide of this "C_{26}-bile alcohol" in the urine of healthy subjects (0.12 mg/24 hr) and patients with liver disease (2 mg/24 hr).

In other studies, Karlaganis et al. (36) showed that 26 (or 27)-nor-5β-cholestane-3α,7α,12α,24ξ,25ξ-pentol was produced from cholesterol and not sitosterol. Although the physiologic importance of the nor-pentol is unknown, it is possible that it is a by-product of bile acid synthesis. Perhaps the high concentrations of plant sterols (10, 11) in the plasma of patients with sitosterolemia interfere with the normal production of bile acids from cholesterol and result in the production of this bile alcohol. Since it is found in other liver diseases (24–26), obviously it is not unique in sitosterolemia.

TABLE 3. Circular dichroism of 5β-cholestanepentols[a]

No.	Compound	CD Δε[b]	CD λ, nm	Chirality[c]
1	5β-Cholestane-3α,7α,12α,24R,25-pentol[d]	− 13.5	309	24R
		+ 9.2	285[e]	
2	5β-Cholestane-3α,7α,12α,24S,25-pentol[d]	+ 9.5	308	24S
		− 5.9	283[e]	
3	26 (or 27)-Nor-5β-cholestane-3α,7α,12α,24S,25-pentol[f]	− 1.97	310	24S
		+ 1.67	285[e]	

[a]CD spectra were performed in $CHCl_3$ solution with the molar ratio of substrate: $Eu(fod)_3$, 1:1.
[b]The Δε is the coefficient of dichroic absorption and is expressed by D/Cl, where D is the observed difference in the values of absorbance between left and right circular, polarized light, C is the molar concentration, and l is the path length of the cell in cm.
[c]The conformer with the bulkier groups to the rear is used to define the chirality of acyclic glycols.
[d]Synthetic (16).
[e]A second Cotton effect of opposite sign is observed around 290 nm.
[f]Isolated from the feces of a patient with sitosterolemia and xanthomatosis.

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