CONFIGURATIONAL ASSIGNMENT OF 5β-CHOLESTANE-3α,7α,12α,23,25-PENTOL EXCRETED BY PATIENTS WITH CEREBROTENDINOUS XANTHOMATOSIS (A CIRCULAR DICHROISM STUDY)*

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Received 1-8-79

ABSTRACT

The absolute configuration of the C_{27} pentahydroxy bile alcohol present in bile and feces of two patients with cerebrotendinous xanthomatosis (CTX) was determined by circular dichroism (CD) spectroscopy. Under anhydrous conditions CD spectra of 5β-cholestane-3α,7α,12α,23,25-pentol in the presence of Eu(fod)$_3$ [tris(1,1,1,2,2,3,3-hepta fluoro-7,7-dimethyl-octane-4,6-dionato) europium (III)] exhibited a large induced split Cotton effect at ca. 310 nm. From the induced circular dichroism of 5β-cholestane-3α,7α,12α,23,25-pentol with Eu(fod)$_3$ it was concluded that the CTX bile alcohol has the 1,3 glycol structure with carbon 23 having the R configuration. This information will be useful in elucidating a structural mechanism for the conversion of 5β-cholestanepentols into bile acids in man and rat.

INTRODUCTION

In patients with the rare sterol storage disease cerebrotendinous xanthomatosis (CTX) the primary biochemical defect is abnormal bile acid synthesis (1,2) and as a consequence large amounts of hitherto unrecognized compounds have been found in the bile and feces (2). These unknown compounds have been conclusively identified by chemical synthesis and their structures determined by various spectroscopic methods as 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α,7α,12α,24ξ, 25-pentol and 5β-cholestane-3α,7α,12α,23ξ,25-pentol (3,4,5). The currently accepted pathway of cholic acid biosynthesis from cholesterol does not include 25-hydroxylated intermediates, and side chain cleavage is thought to proceed via

26-hydroxylation of 5β-cholestane-3α,7α,12α-triol (6). Recently an alternate pathway of cholic acid biosynthesis involving bile alcohols hydroxylated at carbon 25 was discovered in our laboratory (7). The enzymes involved were found to be stereospecific with respect to both hydroxylation at carbon 24 and cleavage of the side chain.

The insertion of hydroxyl groups into the 23 or 24 position of 5β-cholestane-3α,7α,12α,25-tetrol was found to be stereospecific. For example, in the case of the two 5β-cholestane-3α,7α,12α,24,25-pentols, epimeric at carbon 24, only 24 "α" compound was formed in bile and feces. The 24 "β" pentol was not detected in bile or feces since it was rapidly converted to cholic acid in vivo and in vitro (7). Only a single epimer of 5β-cholestane-3α,7α,12α,23,25-pentol was detected. While this pentol was the most abundant bile alcohol in one of the CTX patients it seems unlikely that 23-hydroxylated compounds can serve as precursors of the normally occuring bile acids.

In a previous publication configurational assignment at carbon 24 for 5β-cholestane-3α,7α,12α, 24(α and β), 25-pentols has been made by us from molecular rotation measurements (4). And the absolute stereochemistry of these compounds at carbon 24 (24α=24R, 24β=24S) was determined by circular dichroism studies (8).

The present study describes the absolute configuration at carbon 23 of 5β-cholestane-3α,7α,12α,23,25-pentol via CD spectroscopy.

MATERIALS AND METHODS

Physical measurements: Melting points were determined on a Thermolyne apparatus, model MP-12600, and are uncorrected.

Optical rotations were determined at 25°C in methanol on a Carey model 60 spectropolarimeter.

GLC: The bile alcohols, as the TMSi-derivatives, were analyzed on a 180 cm x 4 mm column packed with either 3% QF-1 or 1% HI-EFF 8BP on 80/100 mesh Gas Chrom Q; column temp. 230°C (Hewlett-Packare model 7610 gas chromatograph).

Mass spectra of the bile alcohols were obtained with a Varian MAT-111 gas chromatograph-mass spectrometer (Varian Associates, Palo Alto, Ca.). High resolution mass spectra were recorded on a model CEC-110 (Consolidated Electrodynamics Corp., Monrovia, Ca.).

TLC: The bile alcohols were separated on silica gel G plates (Brinkman, 0.25 mm thickness). The spots were detected with phosphomolybdic acid (3.5% in isopropanol), sulphuric acid (10%) and heating for one minute at 110°C. Bands on preparative TLC were made visible with iodine.

Eu(fod)$_3$: Tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octane-4,6-dionato) europium (III) (Thompson-Packard, Fort Lee, N.J.) was used as a complexing agent without further purification.

Circular Dichroism: The circular dichroism measurements were carried out on a Jasco J-20 instrument. A 1:1 mixture of the bile alcohol and complex Eu(fod)$_3$ was made in dry chloroform (ethanol free) so that the concentration of the solutes was 2 x 10^{-4} M. The circular dichroism was measured after 30-60 minutes at 24°C, under a stream of high purity dry N_2 with a cell thickness of 0.1 cm. The coefficient of dichroic absorbtion, $\Delta\varepsilon$ was calculated from the molar ellipticity (θ) by the following equation: Molar ellipticity $\theta = 3300\ \Delta\varepsilon$(8,9). Both the molar ellipticity θ and the $\Delta\varepsilon$ are expressed in degree x cm^2 x dmol^{-1}. The Cotton effect was measured at its maxium value, around 310 nm, and was found to correlate with the chirality of the two hydroxy groups (10).

Isolation of 5β-cholestane-3α,7α,12α,23α,25-pentol and 5β-cholestane-3α,7α,12α,24α,25-pentol (Fig. 1, III and I): 5β-cholestane-3α,7α,12α,23α,25-pentol (m.p. 210-211°C) and 5β-cholestane-3α,7α,12α,24α,25-pentol were isolated from the bile and feces of two patients with CTX (2,4). The compounds were purified by thin-layer chromatography (Table 1), and crystallized from ethyl acetate:methanol as previously

described (3,4).

Preparation of 5β-cholestane-3α,7α,12α,24α,25-pentol and 5β-cholestane-3α,7α,12α,24β,25-pentol (fig. 1, I and II). 5β-cholestane-3α,7α,12α,24α,25-pentol (m.p. 212-214°C) and 5β-cholestane-3α,7α,12α,24β,25-pentol (m.p. 213-205°C) were synthesized from cholic acid and purified as described by Dayal, et.al. (Table 1) (3). Cholic acid was converted into its higher homologue, homocholic acid, by the Arndt-Eistert method which on further treatment with diazomethane gave methyl homocholate. A Grignard reaction of methyl magnesium iodide with methyl homocholate yielded 5β-cholestane-3α,7α,12α,25-tetrol (m.p. 189-191°C), which was dehydrated to form a mixture of 5β-cholest-24-ene-3α,7α,12α-triol and the corresponding Δ^{25} compound. Oxidation of the Δ^{24} compound with OsO_4 yielded 5β-cholestane-3α,7α,12α,24,25-pentol. This mixture of two pentols epimeric at carbon 24 was separated by thin layer chromatography (3).

RESULTS AND DISCUSSION

In the bile and feces of the two CTX patients studied, the pentahydroxy bile alcohol fraction consisted of two major components. The predominant bile alcohol of the pentol fraction was 5β-cholestane-3α,7α,12α,23ξ,25-pentol, amounting to approximately 80% by weight, while 5β-cholestane-3α,7α,12α,24α,25-pentol accounted for approximately 20% of this fraction. The less abundant pentol was shown to be identical with 5β-cholestane-3α,7α,12α,24α,25-pentol, which had been prepared form 5β-cholestane-3α,7α,12α,25-tetrol (3). In Table 1 are listed the physical properties of 5β-cholestane-3α,7α,12α,24α,25-pentol, 5β-cholestane-3α,7α,12α,24β,25-pentol and biosynthetic 5β-cholestane-3α,7α,12α,23ξ,25-pentol. The purity of each compound was established by m.p., TLC, GLC, and MS (3,4).

The tentative configurational assignment at carbon 24 for 5β-cholestane-3α,7α,12α,24(α and β), 25-pentols had al-

ready been made by us from molecular rotation measurements (4) (Table 1) and the chirality at carbon 24 established by circular dichroism (CD) studies employing Nakanishi's method for vicinal glycols (1,2 and 1,3 systems) (Table 2), (8,9).

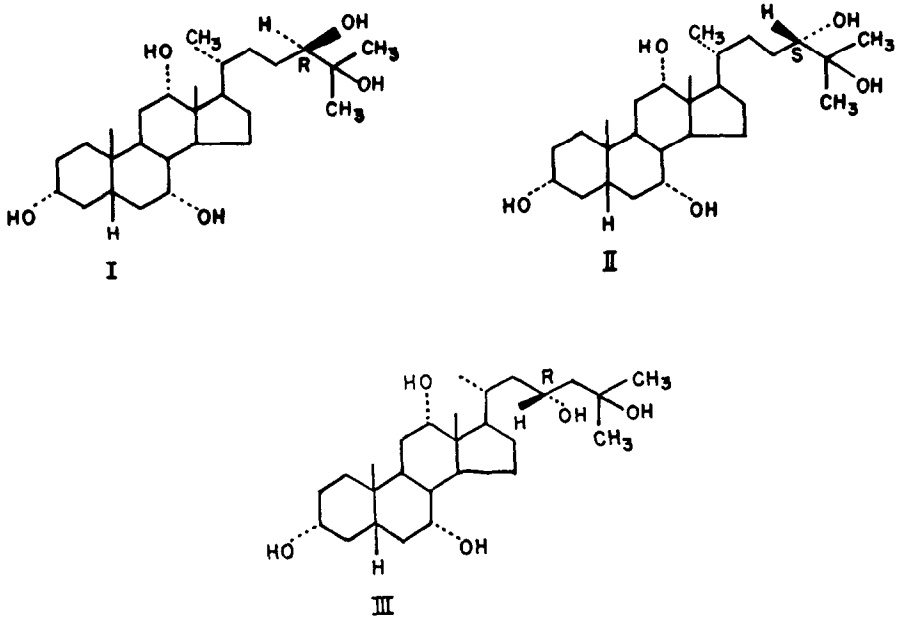

Fig.1

For the 5β-cholestanepentols epimeric at carbon 24, it was observed that the 24α-pentol showed a negative induced Cotton effect ($\Delta\varepsilon_{308} \cong -13.5 \pm 1$ degree x cm^2 x $dmol^{-1}$); while the 24β-pentol showed a positive induced Cotton effect ($\Delta\varepsilon_{308} = +9.5 \pm 1$ degree x cm^2 x $dmol^{-1}$) (Table 2) (8). These observations, according to Nakanishi's empirical rule for

predicting chirality, define the chirality at carbon 24 as R for the 24α,25-pentol (1,2 glycol system) with a negative Cotton effect and S for the 24β,25-pentol with a positive Cotton effect.

When our biosynthetic 5β-cholestane-3α,7α,12α,23,25-pentol (Fig. 2) was examined by CD spectroscopy in the presence of $Eu(fod)_3$, large induced split Cotton effects were obtained around 310 nm, due to the formation of a bidentate adduct between the glycol (23,25-pentol equivalent to a 1,3 glycol system) and europium complex. The results of CD measurements for 5β-cholestane-3α,7α,12α,23α,25-pentol showed a $\Delta\varepsilon_{320}$ value of -2.7 degree x cm^2 x $dmol^{-1}$ (Fig. 2).

The chirality of the 23,25-pentol was then defined as being negative (Fig. 2) or positive, respectively when the Newman projection represents a counterclockwise (left handedness) or clockwise (right handedness) rotation from one hydroxyl group to the other. Mixtures of the 23,25-pentol and complex $Eu(fod)_3$ resulted in CD curves having two Cotton effects of opposite signs at ca. 310 and 290 nm. The amplitudes of these Cotton effects have been found to be dependent on several factors but the sign of the longer wavelength called the first Cotton effect coincided with the chirality of the respective glycols (either 1,2 or 1,3).

Thus in the biosynthetic 5β-cholestane-3α,7α,12α,23α,25-pentol (equivalent to a 1,3 glycol system) the negative induced Cotton effect obtained at ca. 310 nm defined the chirality at carbon 23 as R.

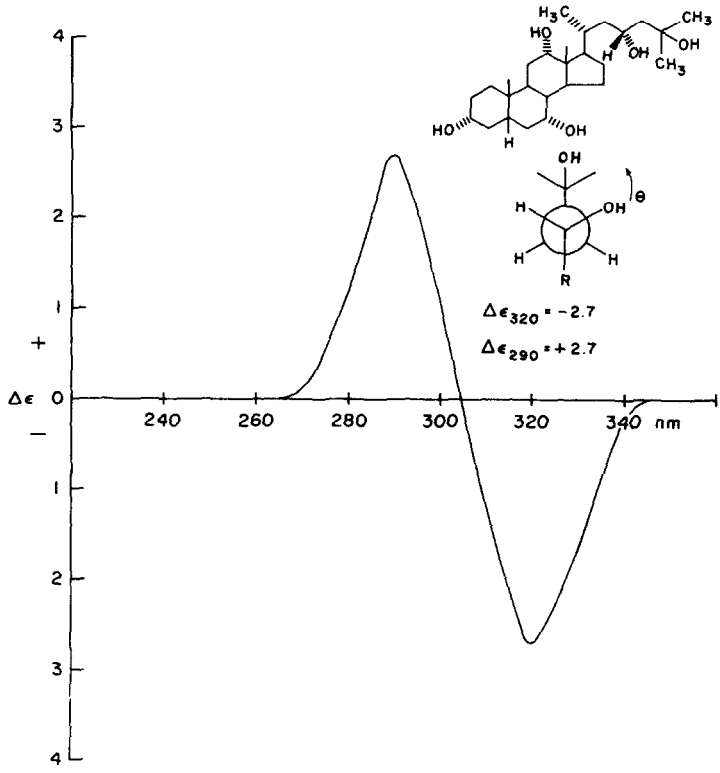

Figure 2. Circular dichroism of 2 x 10^{-4} M 5β-cholestane-3α,7α,12α,23α,25-pentol and 2 x 10^{-4} M $Eu(fod)_3$ in dry $CHCl_3$ at ambient temperature within 35 minutes after mixing.

TABLE 1: PHYSICAL PROPERTIES OF THE ISOMERIC PENTOLS

	5β-Cholestane-3α,7α,12α,24α,25-pentol	5β-Cholestane-3α,7α,12α,24β,25-pentol	5β-cholestane-3α,7α,12α,23ξ,25-pentol
m.p.	212-214°	203-205°	210-211°
TLC[a]	0.30	0.34	0.32
GLC	4.23[b] (1.47)[c]	4.35[b] (1.55)[c]	3.94[b] (1.32)[c]
$[\alpha]_D^{25}$[d]	+44.8°	+28.7°	+45.7°
$[M]_D$	+203°	+130°	+206.5°

[a]Solvent system: chloroform-acetone-methanol, 35:25:7.5 (v/v/v); silica gel G plates, 0.25 mm thick (Brinkmann).

[b]Retention time of TMSi ethers relative to 5α-cholestane. Column: 3% QF-1, column temp. 230°C, retention time of 5αcholestane 2.95 min.

[c]Column: 1% HI-EFF 8BP, column temp. 230°C, retention time of 5α-cholestane 5.42 min.

[d]Determined in methanol (5β-cholestane-3α,7α,12α,24α,25-pentol, 3.39 mg/ml; 5β-cholestane-3α,7α,12α,24β,25-pentol, 1.62 mg/ml; 5β-cholestane-3α,7α,12α,23α,25-pentol, 2.54 mg/ml).

TABLE 2: CIRCULAR DICHROISM OF THE ISOMERIC PENTOLS

ENTRY	COMPOUND	Origin of Sample	Molar ratio substrate $Eu(fod)_3$	Solvent	CD[a] $\Delta\varepsilon$[b]	CD[a] λ, nm[c]	Chirality
1	5β-Cholestane-3α,7α,12α,24α,25-pentol	a) Isolated from CTX patient b) Synthesized in our laboratory	1:1	$CHCl_3$	-13.5 + 9.2[d]	309 285	24R
2	5β-Cholestane-3α,7α,12α,24β,25-pentol	Synthesized in our laboratory	1.1	$CHCl_3$	+ 9.5 - 5.9[d]	308 283	24S
3	5β-Cholestane-3α,7α,12α,23α,25-pentol	Isolated from CTX patient	1:1	$CHCl_3$	- 2.7 + 2.7[d]	320 290	23R

[a]The sign of the longer wave length Cotton effect (first Cotton effect) is in agreement with the chirality of the acyclic α-glycol.

[b]The $\Delta\varepsilon$ is the coefficient of dichroic absorbtion and is expressed by D/C l., 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]A second Cotton effect of opposite sign is observed around 290 nm.

The significance of these data relates to the possible use of these bile alcohols as precursors of bile acids in man and other animals. In the rare inherited sterol storage disease, cerebrotendinous xanthomatosis (CTX), bile acid production is subnormal (1) but considerable quantities of C_{27} bile alcohols are excreted in bile and feces namely, 5β-cholestane-3α,7α,12α,25-tetrol and both 5β-cholestane-3α, 7α,12α,24α,25-pentol and 5β-cholestane-3α,7α,12α,23α,25-pentol (2,4). Although all three compounds are potential precursors of bile acids, we have demonstrated by in vitro and in vivo experiments that only 5β-cholestane-3α,7α,12α,25-tetrol was converted to cholic acid (11). The reaction sequence involves the stereospecific formation of a 24β-hydroxy-pentol, 5β-cholestane-3α,7α,12α,24β,25-pentol, which is in turn transformed to cholic acid (7). Thus, only the 5β-cholestane-3α,7α,12α,24β,25-pentol (24S) is capable of being acted upon by the hepatic enzymes that catalyse side-chain cleavage. The 5β-cholestane-3α,7α,12α,24α,25-pentol (24R) and the 5β-cholestane-3α,7α,12α,23ξ,25-pentol (23R) apparently do not have the structure to interact with the appropiate dehydrogenase or hydroxylase in this pathway.

Recently, Hoshita, et. al., reported the synthesis of the epimeric 5β-cholestane-3α,7α,12α,23,25-pentols (23R and 23S) (12). One of the epimers was obtained in crystalline form and this compound was identical with the material isolated from bile and feces of CTX patients with respect to MS, m.p. $[\alpha]_D$ and migration on TLC. Hoshita, et. al., assigned to

this compound the 23S configuration by comparing its molecular rotation with those of the epimeric 23-hydroxy-lanosterols (23R and 23S) (12). However, the assignment of the 23S configuration to the biosynthetic pentol must be considered inconclusive since the molecular rotation of only one of the epimers was determined and since CD spectra were not previously reported. The circular dichroism studies presented in this paper lead us to conclude that the configuration of the biosynthetic 5β-cholestane-3α,7α,12α,23α,25-pentol is 23R.

ACKNOWLEDGMENTS

We are indebted to Mr. Jeffrey Speck for his skillful technical assistance. This work was supported in part by U.S. Public Health Service Grants AM-18707, HL-17818 and AM-19696.

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