Structure and Stereochemistry of the Higher Bile Acid Isolated from Turtle Bile: (22*S*,25*R*)-3α,12α,15α,22-Tetrahydroxy-5β-cholestan-26-oic Acid

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The structure and stereochemistry of the higher bile acid, tetrahydroxyisosterocholanic acid (TISA), which was previously isolated from the bile of *Amyda japonica* (turtle) and proposed as a tetrahydroxyisosterocholanic acid, have been established as (22*S*,25*R*)-3α,12α,15α,22-tetrahydroxy-5β-cholestan-26-oic acid by X-ray crystallographic analysis of its ethyl ester.

Key words higher bile acid; X-ray analysis; *Amyda japonica* (turtle); steroid; configuration; ethyl (22*S*,25*R*)-3α,12α,15α,22-tetrahydroxy-5β-cholestan-26-oate

The bile of the turtle or the tortoise is known to contain a number of chemically and biologically interesting bile acids and bile alcohols. More than 50 years ago, two bile acid lactones called tetrahydroxysterocholanic lactone (TSL) from the bile of *Amyda* (*A.*) *japonica* (turtle) and tetrahydroxyisosterocholanic lactone (TISL) from the bile of *Emys orbicularis* (tortoise) were isolated by Suganami and Yamasaki,[1] and by Kim,[2] respectively, and were assigned the same chemical formula, $C_{28}H_{46}O_5$. Later, in 1965, Amimoto *et al*.[3] showed in the study of the bile of the turtle (*A. japonica*) that the two lactones mostly exist in unconjugated acid forms, namely tetrahydroxysterocholanic acid (TSA) and tetrahydroxyisosterocholanic acid (TISA), respectively, in the bile and that the largest portion of them could be lactonized to TSL and TISL, respectively, by treatment with hydrochloric acid (HCl) during the course of the extraction. Amimoto *et al*.[3] also suggested that the chemical constitution of TSL is 3α,7α,12α,22ζ-tetrahydroxycoprostanic lactone, and that TISL is not a stereoisomer of TSL, but a position isomer in which the position of one hydroxyl group is different. Twenty years thereafter the full structure and configuration of TSL was determined as (22*S*,25*R*)-3α,7α,12α-trihydroxy-5β-cholestano-26,22-lactone on the basis of ^{1}H-NMR data by Fujimoto *et al*.[4] As for TISL or TISA, however, their structures have not yet been fully elucidated in spite of numerous studies. The present paper describes the isolation of TISA, the preparation of its ethyl ester and the establishment of the complete chemical structure of the ethyl ester of TISA by means of X-ray crystallographic analysis.

Results and Discussion

The bile of turtle (*A. japonica*) was collected by extraction of the gallbladder with ethanol. The concentrated ethanolic extract was chromatographed on a PHP-LH-20 column as described previously.[5] The unconjugated bile acid obtained was esterified with 2% HCl in ethanol at room temperature for 4 h. The crude reaction product was purified by column chromatography followed by repeated crystallization and gave colorless crystals of **1** (see Experimental).

Single crystals of **1** suitable for X-ray crystallographic analysis were slowly grown in ethanol solution. A colorless thin plate crystal having approximate dimensions of 0.30×0.20×0.02 mm was mounted on a glass fiber. All X-ray measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated CuK_α radiation and a rotating anode generator. The crystal data are as follows: $C_{29}H_{50}O_6$, M=494.7, monoclinic, space group $P2_1$(#4), a=10.390(4) Å, b=7.631(3) Å, c=17.668(4) Å, β=99.77(3)°, V=1380.5(8) Å^3, Z=2, D_{calc}=1.190 g·cm^{-3}, μ(CuK_α)=6.48 cm^{-1}, F(000)=544. Cell constants and an orientation matrix for data collection were obtained from a least-squares refinement using the setting angles of 11 carefully centered reflections in the range $42.59<2\theta<44.31°$. Intensity data were collected at 23 °C using the ω–2θ scan technique to a maximum 2θ value of 110.2°. A total of 1974 reflections were collected, of which 1902 were unique. The crystal structure was solved by direct methods (SIR92[6]) and expanded using Fourier techniques (DIRDIF94[7]). The non-hydrogen atoms were refined anisotropically. The hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 1166 observed reflections ($I>3.00\sigma(I)$) and 317 variable parameters, and converged with unweighted and weighted agreement factors of R=0.054 and R_w=0.078, respectively. All calculations were performed using the teXsan program.[8] The final positional and thermal parameters for all non-hydrogen atoms (atomic numbering is shown in Fig. 2) are given in Table 1. An ORTEP drawing of the molecule of **1** here obtained is shown in Fig. 2, where an enantiomer is chosen matching the absolute stereostructure of natural bile acids. Thus, the complete structure and the configuration of **1** have been elucidated as ethyl (22*S*,25*R*)-3α,12α,15α,22-tetrahydroxy-5β-cholestan-26-oate as shown in Fig. 1.

As for the stereochemistry of the side chain, this compound **1** and TSA have the same configuration at C-22 and C-25 (22*S* and 25*R*). In their steroid nucleus, however, **1** has a unique structure possessing 15α-hydroxyl group instead of 7α-hydroxyl group, whereas TSA has 7α-hydroxyl but not 15α-hydroxyl group.[4]

In the case of C_{24} bile acids, it is widely known that 7-dehydroxylation is caused by the microorganisms during enterohepatic circulation; for example, cholic and chenodeoxy-

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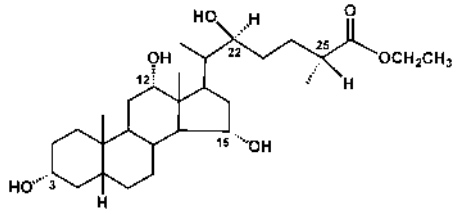

Fig. 1. Structure of Ethyl (22*S*,25*R*)-3α,12α,15α,22-Tetrahydroxy-5β-cholestan-26-oate

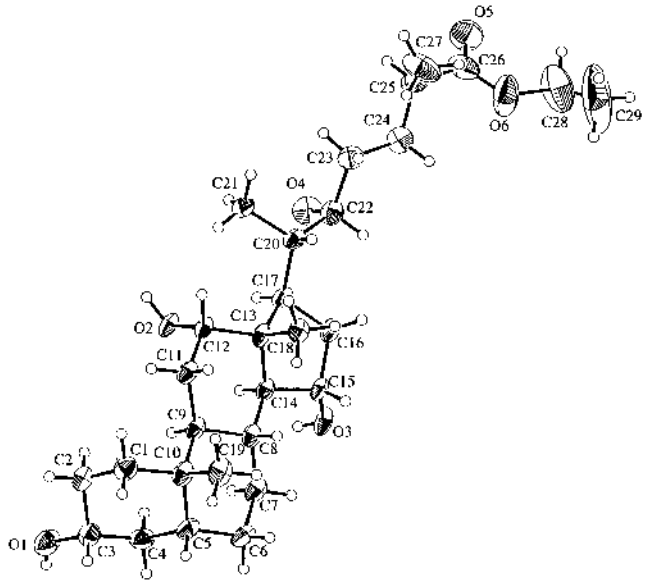

Fig. 2. X-Ray Crystallographic Structure of the Compound **1**

cholic acids are converted to deoxycholic and lithocholic acids, respectively. Thus, this compound **1** was suggested to be formed after the loss of 7α-hydroxyl group by the microorganisms during enterohepatic circulation in the same manner as the C_{24} bile acid. In this study, we also found 3α,12α,22-trihydroxy-5β-cholestan-26-oic acid as a minor constituent in the bile of *A. japonica* (unpublished data).

On the other hand, only one bile acid from a natural source possessing 15α-hydroxyl group was found in the bile of the wambat,[9] and this was identified as 3α,15α-dihydroxy-5β-cholan-24-oic acid. But it was not clear in the literature whether this 15α-hydroxylation occurred in the liver or was caused by microorganisms during enterohepatic circulation. In another experiment,[10] when the sulfonate analogue of bishomochenodeoxycholate, 3α,7α-dihydroxy-25,26-bishomo-5β-cholane-26-sulfonate, was administered to a conventional hamster, the 15α-hydroxylated compound, 3α,7α,15α-trihydroxy-25,26-bishomo-5β-cholane-26-sulfonate, was isolated as a major metabolite from the feces. It was also shown that this 15α-hydroxyl compound was detected in the liver of a hamster which had been treated with antibiotics, suggesting that this 15α-hydroxylation occurred in the liver and was not caused by microorganisms.

From these data, we presumed that TSA was converted to the 7α-dehydroxylated compound by microorganisms during enterohepatic circulation, and that this compound was transformed to TISA by 15α-hydroxylation in the liver after being reabsorbed from the intestine. However, further study is needed to identify the mechanism by which this occurs because the animal is different from those above experiments.

Table 1. Non-hydrogen Atom Fractional Coordinates and Equivalent Isotropic Thermal Parameters for **1**, with Estimated Standard Deviations in Parentheses

Atom	*x*	*y*	*z*	B_{eq} (Å^2)
O(1)	−0.2276 (6)	0.486 (2)	−0.3244 (4)	7.3 (2)
O(2)	0.1249 (4)	0.410 (2)	0.0004 (3)	3.9 (1)
O(3)	0.1240 (5)	1.057 (2)	0.0301 (4)	4.6 (1)
O(4)	0.2718 (5)	0.647 (2)	0.2731 (4)	6.1 (2)
O(5)	0.6395 (9)	0.739 (2)	0.5691 (5)	9.1 (2)
O(6)	0.721 (1)	0.898 (2)	0.4855 (5)	11.1 (3)
C(1)	0.1334 (8)	0.443 (2)	−0.2731 (5)	4.8 (2)
C(2)	−0.0082 (9)	0.394 (2)	−0.2789 (5)	5.1 (2)
C(3)	−0.0945 (8)	0.539 (2)	−0.3199 (5)	5.2 (2)
C(4)	−0.0674 (8)	0.707 (2)	−0.2765 (5)	4.9 (2)
C(5)	0.0785 (8)	0.761 (2)	−0.2703 (5)	4.5 (2)
C(6)	0.1042 (8)	0.940 (2)	−0.2295 (5)	5.1 (2)
C(7)	0.1026 (8)	0.928 (2)	−0.1438 (5)	4.6 (2)
C(8)	0.1985 (7)	0.788 (2)	−0.1057 (5)	3.7 (1)
C(9)	0.1672 (6)	0.610 (2)	−0.1451 (5)	3.6 (1)
C(10)	0.1732 (7)	0.619 (2)	−0.2321 (5)	4.3 (1)
C(11)	0.2484 (7)	0.461 (2)	−0.1027 (5)	4.0 (2)
C(12)	0.2505 (6)	0.457 (2)	−0.0158 (5)	3.4 (1)
C(13)	0.2929 (6)	0.635 (2)	0.0199 (5)	3.4 (1)
C(14)	0.1996 (6)	0.775 (2)	−0.0192 (4)	3.3 (1)
C(15)	0.2307 (6)	0.937 (2)	0.0302 (5)	3.5 (1)
C(16)	0.2841 (8)	0.866 (2)	0.1098 (5)	4.1 (2)
C(17)	0.2814 (6)	0.666 (2)	0.1066 (5)	3.5 (1)
C(18)	0.4367 (6)	0.669 (2)	0.0092 (5)	3.9 (2)
C(19)	0.3133 (8)	0.658 (2)	−0.2462 (5)	5.4 (2)
C(20)	0.3812 (7)	0.572 (2)	0.1681 (5)	3.9 (1)
C(21)	0.3493 (8)	0.378 (2)	0.1723 (5)	4.8 (2)
C(22)	0.3941 (7)	0.658 (2)	0.2465 (5)	4.7 (2)
C(23)	0.5023 (8)	0.588 (2)	0.3065 (5)	5.1 (2)
C(24)	0.5416 (8)	0.707 (2)	0.3731 (5)	5.3 (2)
C(25)	0.632 (1)	0.629 (2)	0.4421 (6)	6.4 (2)
C(26)	0.666 (1)	0.755 (3)	0.5051 (7)	7.1 (2)
C(27)	0.758 (1)	0.558 (3)	0.4179 (6)	8.9 (3)
C(28)	0.759 (2)	1.041 (4)	0.539 (1)	14.3 (7)
C(29)	0.859 (3)	1.126 (5)	0.526 (1)	25.0 (1)

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and were uncorrected. ^{1}H-NMR spectra were recorded on a JEOL GX-400 (400 MHz) spectrometer with tetramethylsilane as an internal standard. Piperidinohydroxypropyl-Sephadex-LH 20 (PHP-LH-20) was prepared as described previously.[5] TLC was performed on precoated silica gel sheets (Merck Silica gel 60, 0.2 mm thickness) and the spots were detected by spraying with10% phosphomolybdic acid in ethanol followed by heating. Silica gel used for column chromatography was Silica gel 60 (Merck, 0.063—0.200 mm).

Material Gallbladders, obtained from the turtle *A. japonica* caught in 1994, in Hiroshima, Japan, were homogenized and the mixture was freeze-dried. This material was used for the isolation of the bile acid.

Isolation of Ethyl TISA The bile of *A. japonica* (turtle) was collected by extraction of 60 gallbladders with 700 ml of ethanol. The ethanolic extract was evaporated to dryness under reduced pressure. The crude extract (2.9 g) was dissolved in 90% ethanol and applied to PHP-LH-20 column (4×40 cm) as described previously.[5] The unconjugated bile acid was eluted with 0.1 M acetic acid in 90% ethanol and the eluate was evaporated to dryness below 40 °C. The residue (0.53 g) was esterified by adding 50 ml of 2% HCl in ethanol and allowed to stand at room temperature for 4 h. The reaction mixture was diluted with 200 ml of water and extracted with ether (300 ml×2). Evaporation of the solvent gave a residue which was chromatographed on a silica gel (50 g) column using stepwise gradient elution with mixtures of chloroform and ethanol as the eluent. Each fraction was monitored by TLC (solvent, chloroform–ethanol 8 : 2), fractions eluted with chloroform–ethanol (7 : 3) were combined and the solvents were evaporated to dryness below 40 °C. The residue (147 mg) was crystallized from ethyl

acetate and recrystallization from ethanol gave colorless crystals (77.5 mg), mp 225—228 °C. ^{1}H-NMR (CD_3OD) δ: 0.73 (3H, s, 18-CH_3), 0.93 (3H, d, J=7.3 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 1.14 (3H, d, J=7.1 Hz, 27-CH_3), 1.25 (3H, t, J=7.1 Hz, $COOCH_2C\underline{H}_3$), 3.52 (1H, m, 3β-H), 3.56 (1H, m, 22-H), 3.86 (1H, m, 15β-H), 3.88 (1H, m, 12β-H) , 4.10 (1H, dq, J=10.7, 7.1 Hz), 4.14 (1H, dq, J=10.9, 7.1 Hz) (–$COO\underline{CH}_2CH_3$).

Repeated recrystallization from ethanol gave colorless thin plate crystals suitable for X-ray crystallographic analysis.

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