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Major biliary bile acids of the Medaka (*Oryzias latipes*): 25*R*- and 25*S*-epimers of 3α,7α,12α-trihydroxy-5β-cholestanoic Acid

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Abstract

The biliary bile salts of the medaka, the Japanese rice fish (*Oryzias latipes*) were isolated and identified. Only bile acids were present, and all were *N*-acyl amidated with taurine. Three bile acids, constituting 98% of total bile acids, were isolated by chromatography and their structure inferred from their properties compared to those of synthetic standards when analyzed by liquid chromatography-tandem mass spectrometry. The dominant bile acid was the 25*R*-epimer (82%) of 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid. The 25*S*-epimer was also present (11%), as was cholic acid (5%). Complete ^{1}H and ^{13}C NMR signal assignments of the C-25 epimers were made by using a combination of several 1D- and 2D-NMR techniques. The ^{1}H and ^{13}C NMR chemical shifts and spectral patterns of the hydrogen and carbon atoms, being close to the asymmetric centered at C-25, provided confirmatory evidence in that they distinguished the two epimeric diastereomers. The medaka is the first fish species identified as having C_{27} biliary bile acids as dominant its major bile salts.

Keywords

cholic acid; trihydroxycholestanoic acids; higher bile acids; peroxisomes; Acyl CoA-racemase; fish

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Introduction

Bile salts, multifunctional, amphipathic end products of cholesterol metabolism, vary considerably in structure between species (Hagey, 1992; Haslewood, 1967; Hofmann et al., in press; Hoshita, 1985; Une and Hoshita, 1994). Most bile salts may be subdivided into three major classes -- C_{27} bile alcohols, C_{27} bile acids, and C_{24} bile acids -- based on the length of the alkyl side chain and whether the terminal polar group is a primary alcohol or a carboxylic acid (Hofmann et al, in press; Hofmann and Hagey, 2008). Additional structural variation arises from the configuration of the A/B ring juncture (*cis* or *trans*), and the number and orientation of hydroxy groups on the steroid nucleus and side chain. After their synthesis, bile alcohols are esterified with sulfate; C_{27} bile acids are *N*-acylamidated (conjugated) with taurine; and C_{24} bile acids, with taurine or glycine. Such conjugation converts bile acids and bile alcohols to bile salts that are soluble at intestinal pH and renders them impermeant to cell membranes, thereby promoting a high, micellar intraluminal concentration which facilitates lipid absorption (Hofmann and Hagey, 2008).

In a recent review compiled by Hofmann et al (in press), available information on biliary bile salt structures from about 700 vertebrate species-fish, reptiles (turtles, crocodilians, squamates), birds, and mammals was tabulated. In early evolving fish and amphibians, C_{27} bile alcohols dominated; in most later evolving fish the common C_{24} 5β- bile acids, chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) and cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) were the major bile acids. C_{27} bile acids prevailed in reptiles and early evolving birds (emus, kiwis, ostriches, tinamous). C_{24} bile acids occurred in all vertebrate classes, but were most predominant in mammals, including humans. Bile salt composition showed significant variation between orders and not between the smaller divisions of families, genera, or species. Thus biliary bile salt composition appears to be a biochemical trait that provides clues to evolutionary relationships, complementing anatomical and genetic analyses.

One unexpected finding in the survey of bile salts in fish was that the biliary bile salts of the "Japanese rice fish" (*Oryzias latipes*), often referred to by its Japanese name "medaka", differed other fish species in having a high proportion of C_{27} bile acids.

The Japanese rice fish is a small, Southeast Asian pond fish that has been a popular aquarium fish for several centuries (Parenti, 2008). Its genome has been sequenced (Kasahara et al., 2007), and the fish is widely used as a model organism in many areas of biological research (Matsumoto et al., 2009). We judged it of interest to determine the exact structure of the C_{27} bile acids occurring in the medaka, not only because it is the only fish species identified to data in whom C_{27} acids predominate, but also because the bile acid composition of the medaka might be interpreted in light of the genes involved in bile acid evolution. A photograph of this fish species, which in this paper will be referred to as the medaka, is shown in Figure 1.

We report here that the major biliary bile acids of the medaka consist of the taurine conjugates of 25*R*- and 25*S*-epimers of 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid. We also show that the ^{1}H and ^{13}C NMR spectra of the two epimers are sufficiently different that the two taurine-conjugated epimers can be distinguished using this analytical technique.

Material and methods

Biological material

Gallbladder bile of the Japanese rice fish (*Oryzias latipes subsp*.) was collected by excising the gallbladder from about 1000 medaka fish. Bile samples were dispersed in 4 volumes of reagent-grade isopropanol and kept at 4°C until analysis.

Material and reagents

Authentic reference compounds of the taurine conjugates of CA and (25*R*)- and (25*S*)-3α,7α, 12α-trihydroxy-5β-cholestan-27-oic acid (THCA) were synthesized in our laboratory (Goto et al., 1989b; Une et al., 1984).

RP-HPLC analysis of gallbladder bile of the medaka

The RP-HPLC apparatus used was a Jasco LC-2000 plus HPLC system (two PU-2085 high-pressure pumps, an MX-2080-32 solvent mixing module, and a CO-2060 column heater) equipped with a ChromNAV data-processing system (Tokyo, Japan). A Capcell Pack type AQ C_{18} column (3.0 mm × 150 mm I.D.; particle size, 3 μm; Shiseido) was employed and kept at 37°C. An Alltech 2000ES evaporative light-scattering detector (ELSD) (Deerfield, IL, USA) was used under the following conditions; the flow rate of purified compressed air used as a nebulizing gas was 2.0 L/min, and the temperature of the heated drift was 82°C. The mobile phase used was 20 mM-ammonium acetate/acetic acid buffer solution (pH 3.8)-methanol mixture (7:3, v/v); the flow rate was kept at 400 μL/min during the analysis.

Isolation of major bile salts from the bile of Japanese rice fish

The isopropanol solution of medaka bile was ultracentrifuged for 10 min (20,000 rpm) and the supernatant liquid was filtered with Mini-Uni Prep membrane filter (pore size, 0.45 μm; Whatman, NJ, USA). The filtrate was passed through a pre-conditioned Sep-Pak tC_{18} cartridge (5 g; Waters, Milford, MA). After the cartridge was washed successively with water (5 mL) and 5% methanol (5 mL), the bile salt fraction was eluted with methanol (5 mL). The methanol eluate was concentrated under a nitrogen stream at 40°C and the major bile salts were isolated by preparative RP-HPLC.

The preparative RP-HPLC apparatus consisted of a Jasco Gulliver series HPLC system with two PU-980 high-pressure pumps, an HG-980-31 solvent mixing module, and an HG-980-50 degasser. RP-HPLC was carried out by stepwise gradient elution using an Inertsil ODS-3 column (5 μm, 250 mm × 20 mm I.D.; GL Science, Tokyo, Japan) using a gradient of methanol in 5 mM-ammonium acetate as the mobile phase. The methanol composition was gradually increased at a flow rate of 7.2 mL/min using the following HPLC conditions: 5% (0–15 min) → 40% (15.1–30 min) → 50% (30.1–45 min) → 60% (45.1–60 min) → 74~92% (150.1–210 min). The 70, 72, and 74–80% methanol fractions, which contained compounds A, C, and D, respectively, were collected. Each of the fractions was evaporated under reduced pressure and then applied to a pre-conditioned Sep-Pak tC_{18} cartridge (360 mg; Waters, Milford, MA). After the cartridge was washed with water (7 mL), each of the isolated compounds was eluted with methanol (5 mL), and the eluate was evaporated under a nitrogen stream at 35°C. Three compounds, termed A, C, and D, were isolated and then analyzed using LC/ESI-MS/MS and 1D- and 2D-NMR techniques.

LC/ESI-MS/MS analysis of major components A, C, and D

Negative ion LC/ESI-MS/MS analysis of the isolated compounds was performed using an API 5000 LC-MS/MS system (Applied Biosystems, Inc., CA) equipped with a Nanoscope HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was carried out with a Capcell Pak C_{18} type MGII column (5 μm, 100 × 2.0 mm ID) using 10 mM-ammonium acetate (pH 7.0)/ methanol mixture (1:1, v/v) as the mobile phase at a flow rate of 200 μL/min. The mass detector was set to the following conditions: curtain gas (N_2) flow, 20 psi; nebulizer gas (N_2) flow, 40 psi; turbo gas (zero grade air) flow, 70 psi; turbo ion spray temperature, 600°C; declustering potential, −70 V; collision energy, −90 eV; collision gas (N_2) pressure, 8×10^{-3} mbar.

^{1}H and ^{13}C NMR analyses of the major isolated compounds C and D

NMR spectra were recorded at 23°C in CD_3OD in a 5 mm tube on a JEOL ECA-600 instrument using 600 MHZ for ^{1}H and 149.4 MHz for ^{13}C. ^{1}H and ^{13}C resonance assignments were made using a combination of two-dimensional (2D) homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}C) shift-correlated techniques, which include ^{1}H-^{1}H correlation spectroscopy (COSY), long-range COSY, ^{1}H-^{1}H nuclear Overhauser effect spectroscopy (NOESY), ^{1}H detected heteronuclear multiple quantum correlation (HMQC), and ^{1}H detected heteronuclear multiple bond correlation (HMBC) experiments. These 2D-NMR spectra were recorded using standard pulse sequences and parameters recommended by the manufacturer. The ^{13}C distortionless enhancement by polarization transfer (DEPT; 135°, 90°, and 45°) spectra were also measured to determine the exact ^{1}H signal multiplicity and to differentiate between CH_3, CH_2, CH, and C based on their proton environments.

Results

Isolation and identification of major bile acids present in medaka bile

As shown in Figure 2, RP-HPLC analysis of the bile salts present in the gallbladder bile of the medaka showed three major peaks, which were designated as compounds A (constituting 5% of total biliary bile salts, C (constituting 11%), and D (constituting 82%). An additional component B was in much lower proportion (less than 2%) and could not be identified. The three major components A, C, and D, were isolated by preparative RP-HPLC.

The individually isolated components A, C, and D were then examined by LC/ESI-MS/MS (Figure 3). In the first ESI-MS spectra, the deprotonated molecules $[M-H]^-$ and $[M-H-H_2O]^-$ were as follows: peak A, *m/z* 514 and 496, indicating a taurine-conjugated C_{24} trihydroxy bile acid; and peaks C and D, *m/z* 556 and 538, indicating taurine-conjugated C_{27} trihydroxy bile acids. In the collision induced dissociation (LC/ESI-MS/MS) spectra obtained by selecting the deprotonated ions as a precursor ion, all three components afforded the characteristic fragment ions at *m/z* 124 $[taurine-H]^-$, 107 $[CH_2=CH-SO_3]^-$, and 80 $[SO_3]^-$, indicating the presence of an *N*-acylamide linkage with taurine on the side chain.

Based on their RP-HPLC retention times and the LC-MS fragmentation patterns compared to those of the synthetic reference standards (Goto et al., 1989; Une et al., 1984), the three components A, C, and D were identified as the taurine-conjugate of cholic acid, (peak A), the taurine conjugate of (25*S*)-THCA (peak C) and the taurine conjugate of (25*R*)-THCA (peak D). The assigned structures were confirmed by NMR (see below). Figure 4 shows the steric structures of the three isolated compounds (A, C, and D). Carbon atoms have been numbered.

Characterization of the epimeric (25*R*)-/(25*S*)-THCAs by NMR

To establish the location and orientation of functional groups present in the isolated compounds, we examined their 1D- and 2D-NMR spectra. We wished to determine whether NMR could be used to distinguish the two epimers of THCA, both of which are key intermediates in C_{24} bile acid biosynthesis. Table 1 shows the complete ^{1}H (600 MHz) and ^{13}C (149.4 MHz) signal assignments for the isolated compounds C and D. Carbon signals were grouped according to their multiplicity using DEPT experiments. Subsequently, individual ^{1}H and ^{13}C signal assignments were made from a combination of several 2D-NMR techniques, including COSY, long-range COSY, NOESY, HMBC, and HMQC. Both C-25 epimers showed essentially identical ^{1}H and ^{13}C chemical shifts and signal multiplicities for protons and carbons C-1 to C-19 in the 5β-steroid nucleus (Ijare et al., 2005;Nagane Gowda et al., 2006), because these carbon atoms are far from the chiral C-25.

The assignments of the individual ^{1}H signals to the side chain carbons (C-20~C-29) were based on our previous ^{13}C signal assignments for the taurine conjugate of (25*R*)-THCA (Hagey et al., 2009). The resonance position of these ^{1}H and ^{13}C signals generally gave chemical shifts that were similar for the two epimers. However, careful comparison of both the spectra revealed that some of the side chain protons and carbons, being close to the asymmetric center at C-25, had small, but distinct and significant differences in the chemical shifts and/or signal patterns. Thus, the ^{13}C signals due to C-23 and C-26 exhibited downfield shifts of 0.11 and 0.23 ppm, respectively, for the 25*R*-epimer (compound D) compared to the 25*S*-epimer (compound C), whereas the oxygenated carbon at C-27 showed an upfield shift of 0.10 ppm. Of further interest were the differences in the spectral pattern of the ^{13}C signals arising from C-21 *vs.* C-26 and C-20 *vs.* C-22, as shown in Figure 5. The significant differences in the ^{13}C chemical shifts (defined as Δ-values) between the two epimeric pairs are of particularly useful to characterizing each of the compounds: the Δ-values observed for C-21 *vs.* C-26 is 0.27 ppm in C and 0.00 ppm in D and those for C-20 *vs.* C-22 is 0.10 ppm in C and 0.04 ppm in D.

The ^{1}H signals arising from the hydrogens on the taurine moiety (28-H_2 and 29-H_2) also provided distinct differences in the two 25-epimers. As shown in Figure 6, the ^{1}H signal of 28-H_2 in the 25*S*-epimer (C) occurred at 3.584 ppm as a triplet (*J*, 7.2 Hz), whereas the corresponding ^{1}H signals in the 25*R*-isomer (D) appeared at 3.582 and 3.585 ppm as a double triplet (each *J*, 7.2 Hz). In addition, the ^{1}H signals of 29-H_2 in C appearing at 2.948 and 2.956 ppm exhibited a double triplet with the *J* value of 7.2 Hz, whereas those for D resonated at 2.950 and 2.959 ppm with the *J* values of 6.9 and 7.2 Hz, respectively. These ^{1}H and ^{13}C NMR characteristics can, therefore, be used to distinguish the two C-25 epimeric THCAs (in the form of their taurine conjugates).

Discussion

Chemistry

The analyses reported here indicate that the major bile salts of the medaka are the 25*R*- and 25*S*-epimers of THCA in a ratio of 7 to 1.

In 1939, Kurauti and Kazuno isolated a THCA from the bile of the bullfrog (1939). Two years later, Mabuti (1941) isolated a C_{27} trihydroxy bile acid from bullfrog bile that was similar, but not identical to that isolated by Kurauti and Kazuno (1939). The bile acid isolated by Mabuti was eventually shown to be the *S*-isomer of THCA by Bridgwater, working in the laboratory of Haslewood, who synthesized both epimers (Bridgwater, 1956). Others have improved the synthesis (Briggs, 1970; Kurosawa et al., 1995; Starchenkov et al., 2000) or separation (Batta et al., 1991; Goto et al., 1989a, b; Une et al., 1983) of the two epimers. The exact stereochemical configuration of the *S*-epimer of THCA was established using X-ray crystallography by Batta *et al* (1979a). The concurrent presence of the two epimers in bullfrog and alligator bile has been reported (Une and Hoshita, 1994), and they are also present in the bile of some children with peroxisomal disease (Une et al., 1987). Nonetheless, the view at present is that the C_{27} bile acids occurring in crocodiles, turtles, and squamates, and early evolving birds consist mostly of the *R*-epimer (Une and Hoshita, 1994), although there is little experimental verification of this opinion. We have recently reported that the major C_{27} bile acids present in the bile of the Red-winged tinamou (*Rhynchotus rufescens*), an early evolving bird, consist entirely of the *R*-epimer (Hagey et al., 2009).

A great variety of bile acids with complex side chains occur in amphibians, usually as taurine-conjugates (Une and Hoshita, 1994). Although the amide bond of taurine conjugated THCA can be cleaved enzymatically (Batta et al., 1979b), no such hydrolase is available at present. Concentrated alkali can be used for deconjugation, but may induce artifacts (Une and Hoshita, 1994), although a microwave process has been claimed to effect deconjugation without

racemization (Dayal and Ertel, 1998). Our finding that NMR can be used to distinguish the *R*- and *S*-epimers of THCA suggests that ^{1}H and ^{13}C NMR will prove to be of value in elucidating the exact chemical structure of bile salts with complex side chains. The observed NMR differences between the *R*- and *S*-isomers are probably due to the epimeric diastereomers at C-25.

Bile acid biosynthesis in peroxisomes

It is well known that in mammals, the oxidative cleavage of the *iso*-octane side chain of cholesterol proceeds by a pathway that is essentially identical to β-oxidation of fatty acids and occurs in the peroxisomes (Ferdinandusse et al., 2009). In the neutral biosynthetic pathway of C_{24} bile acids, 5β-cholestane diol or triol undergoes hydroxylation at C-27 (and then oxidation to a carboxyl group) in the mitochondria by sterol 27-hydroxylase (CYP27A). Carboxylation is stereospecific and results in formation of the 25*R*-epimer (Shefer et al., 1978). The CoA ester is then formed. The next step in the β-oxidation pathway is peroxisomal dehydrogenation (oxidation) of the 5β-cholestanoic acid to form (24*E*)-5β-cholest-24-enoic acid mediated by acyl-CoA oxidase. In the desaturation step, there is elimination of the H_R (pro-*R*) at C-24 and H at C-25, and this reaction can only occur with the *S*-epimer of THCA (Ikegawa et al., 1995b, 1998; Van Veldhoven et al., 1996). Therefore, a racemase is required to change the 25*R*-epimer in part to the 25*S*-epimer, and this enzyme has been characterized (Cuebas et al., 2002; Ikegawa et al., 1995a, 1997; Lloyd et al., 2008). These early steps in side chain cleavage of cholesterol to form C_{24} bile acids are illustrated in Figure 7.

It is possible that activity of the racemase is rate-limiting for side chain cleavage in the medaka and other vertebrates whose bile acids contain C_{27} acids. A low racemase activity could explain the predominance of the *R*-epimer of THCA in the medaka, as also occurs in patients with peroxisomal methylacyl-CoA racemase deficiency (Une et al., 1987). The acyl CoA racemase has been sequenced in the medaka and four other fish species (fugu, freshwater puffer fish, rainbow smelt, and zebrafish), and homology compared to the human racemase. In these 5 fish species, the medaka racemase had a lower homology with human racemase (52%) than any of the four other fish species (range 69–71%) (Hagey et al., in press).

Occurrence of C_{27} bile acids in fish

In a recent study, Hagey et al. (2010) determined the biliary bile salt composition of 226 fish species from 38 different orders that included jawless fish (Agnatha), cartilaginous fish, lobe-finned fish, and bony fish. In this survey of fish bile salts, C_{27} bile acids were uncommon and found in species from only five orders: Beloniformes (which includes the medaka), Syngnathiformes, Lophiformes, Gadiformes, and Characiformes. The medaka, however, was the only species of the 226 surveyed for which C_{27} bile acids comprised more than 50% of total biliary bile salts.

Current phylogenetic relationships for fish, based on molecular and morphology data (Nelson, 2006; Kawahara et al., 2008; Lavoué et al., 2008), are shown in Fig 8. The occurrence of C_{27} bile acids in fish from four widely distributed orders in Teleostei suggests the possibility that a common ancestor to these fish used C_{27} bile acids (see nodes in the phylogenetic tree marked with * in Fig. 8). The phenotype of having C_{27} bile acids as a significant fraction of biliary bile acids would represent an ‘ancestral’ trait that is now present in only a small number of fish species. If this hypothesis is correct, then most fish now have the ‘derived’ phenotype of having mainly C_{24} bile acids (the most common bile salt profile in bony fish surveyed so far), with only a small minority of fish species such as the medaka retaining the ancestral phenotype.

Alternatively, a less parsimonious but certainly possible explanation would be that the occurrence of C_{27} bile acids in fish evolved independently in different fish orders. More extensive surveys of biliary bile salt composition in fish species may help in providing evidence for this proposed evolutionary pathway for C_{27} bile acids in fish. The bile salt composition of the medaka differs markedly from that of zebra fish bile, a fish species also used widely for biological research, whose bile salts are composed entirely of 5α-cyprinol (sulfate), a C_{27} bile alcohol (Reschly et al, 2008; Hofmann et al., in press).

Physical and biological properties of C_{27} bile acids

C_{27} bile acids are difficult to isolate in sizable amounts and with high purity from bile, and their synthesis from their C_{24} homologues is difficult. Therefore there has been no systematic comparison of the physicochemical properties of the C_{27} bile acids with those of their corresponding C_{24} homologues. Based on studies of alkyl sulfates or alkyl sulfonates, one would expect the taurine conjugates of C_{27} homologues to have a slightly lower critical micellization concentration and a slightly higher critical micellization temperature than those of their corresponding C_{24} homologues (Shinoda et al, 1963). The presence of branching in the terminal portion of the side chain is likely to diminish the effect of three additional carbon atoms on these parameters. The unconjugated C_{27} bile acids should also have lower aqueous solubilities of the protonated form, and, as a result, they should have higher critical micellization pH values than those of their corresponding C_{24} homologues. In addition, the calcium salts of the unconjugated or glycine conjugated C_{27} bile acid should have lower aqueous solubilities than their C_{24} homologues were they to be present in bile (Hofmann and Mysels, 1992). The toxicity of C_{27} bile acids to cultured cells was recently examined by Ferdinandusse et al (2009). Compared to their C_{24} homologues, C_{27} bile acids were found to be more cytotoxic, and also to impair mitochondrial function, as evidenced by inhibition of ATP formation as well as the generation of reactive oxygen species.

We conclude that the medaka is the first fish species identified to date whose biliary bile salts contain mostly C_{27} bile acids. Low activity of the acyl CoA racemase may contribute to this unusual biliary bile salt composition.

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References

Batta AK, Salen G, Blount JF, Shefer S. Configuration at C-25 in 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by X-ray crystallography. J Lipid Res 1979a;20:935–940. [PubMed: 533828]

Batta AK, Salen G, Cheng FW, Shefer S. Cleavage of the taurine conjugate of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by rat fecal bacteria. J Biol Chem 1979b;254:11907–11909. [PubMed: 500682]

Batta AK, Salen G, Arora R, Shefer S, Batta M. High-performance liquid chromatographic separation of bile acids and bile alcohols diastereoisomeric at C-25. J Chromatogr 1991;542:184–188. [PubMed: 1874839]

Bridgwater RJ. Partial synthesis of the two 3α,7α,12α-trihydroxycoprostanic acids and of similar bile acids with extended side chains. Biochem J 1956;64:593–599. [PubMed: 13382806]

Briggs T. Partial synthesis of 25D- and 25L-cholestanoic acids from some common bile acids. J Org Chem 1970;35:1431–1434. [PubMed: 5440333]

Cuebas DA, Phillips C, Schmitz W, Conzelmann E, Novikov DK. The role of α-methylacyl-CoA racemase in bile acid synthesis. Biochem J 2002;363:801–807. [PubMed: 11964182]

Dayal B, Ertel NH. Rapid hydrolysis of bile acid conjugates using microwaves: retention of absolute stereochemistry in the hydrolysis of (25*R*)-3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine. Lipids 1998;33:333–338. [PubMed: 9560809]

Ferdinandusse S, Denis S, Faust PL, Wanders RJA. Bile acids: the role of peroxisomes. J Lipid Res 2009;50:2139–2147. [PubMed: 19357427]

Ferdinandusse S, Denis S, Dacremont G, Wanders RJ. Toxicity of peroxisomal C_{27}-bile acids intermediates. Mol Genet Metab 2009;96:121–128. [PubMed: 19136287]

Goto J, Gang S, Miura H, Nambara T, Tazawa Y, Tada K. Separation and characterization of C-25 epimers of unconjugated and conjugated trihydroxycholestanoic acids in urine from a patient with Zellweger syndrome by high-performance liquid chromatography. J Liquid Chromatogr 1989a;12:1075–1084.

Goto J, Shao G, Miura H, Nambara T. Separation of C-25 epimers of 5β-cholestanoic acids by high performance liquid chromatography with precolumn fluorescence labeling. Anal Sci 1989b;5:19–22.

Hagey, LR. Bile acid biodiversity in vertebrates: chemistry and evolutionary implication. PhD Dissertation. San Diego: University of California; 1992.

Hagey LR, Kakiyama G, Muto A, Iida T, Mushiake K, Goto T, Mano N, Goto J, Oliveira CA, Hofmann AF. A new, major C27 biliary bile acid in the Red-winged tinamou (Rhynchotus rufescens): (25*R*)-1α,3α,7α-trihydroxy-5β-cholestan-27-oic acid. J Lipid Res 2009a;50:651–657. [PubMed: 19011113]

Hagey LR, Møller PR, Hofmann AF, Krasowski MD. Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. Physiol Biochem Zool. (in press).

Haslewood GAD. Bile salt evolution. J Lipid Res 1967;8:535–550. [PubMed: 4862128]

Hofmann AF, Hagey LR, Krasowski MD. Bile salts of vertebrates: structural variation and possible evolutionary significance. J Lipid Res. (in press).

Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci 2008;65:2461–2483. [PubMed: 18488143]

Hofmann AF, Mysels KJ. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca^{2+} ions. J Lipid Res 1992;33:617–626. [PubMed: 1619357]

Hoshita, T. Bile alcohols and primitive bile acids (Chapter 10). In: Danielsson, H.; Sjövall, J., editors. Sterols and Bile acids. Amsterdam, New York, and Oxford: Elsevier; 1985. p. 279-302.

Ijare OB, Somashekar BS, Jadegoud Y, Nagane Gowda GA. ^{1}H and ^{13}C NMR characterization and stereochemical assignment of bile acids in aqueous media. Lipids 2005;40:1031–1041. [PubMed: 16382575]

Ikegawa S, Goto T, Mano N, Goto J. Substrate specificity of THCA-CoA oxidases from rat liver light mitochondrial fractions on dehydrogenation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid CoA thioester. Steroids 1998;63:603–607. [PubMed: 9830687]

Ikegawa S, Goto T, Watanabe H, Goto J. Stereoisomeric bio-inversion of (25*R*)- and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoic acid CoA thioesters in rat liver peroxisome. Enantiomers 1997;2:333–342.

Ikegawa S, Goto T, Watanabe H, Goto J. Stereoisomeric inversion of (25*R*)- and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoic acids in rat liver peroxisome. Biol Pharm Bull 1995a;18:1027–1029. [PubMed: 7581245]

Ikegawa S, Watanabe H, Goto T, Mano N, Goto J, Nambara T. Stereospecific dehydrogenation of (25*R*)- and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoic acids by acyl-CoA oxidase in rat liver light mitochondrial fraction. Biol Pharm Bull 1995b;18:1041–1044. [PubMed: 8535391]

Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y. The medaka draft genome and insights into vertebrate genome evolution. Nature 2007;447:714–719. [PubMed: 17554307]

Kawahara R, Miya M, Mabuchi K, Lavoué S, Inoue JG, Satoh TP, Kawaguchi A, Nishida M. Interrelationships of the 11 gasterosteiform families (sticklebacks, pipefishes, and their relatives): a new perspective based on whole mitogenome sequences from 75 higher teleosts. Mol Phylogenet Evol 2008;46:224–236. [PubMed: 17709262]

Kurauti Y, Kazuno T. Tetraoxycholan, Trioxycholen und Trioxy-bis-norsterocholansaure aus der Galle von *Rana catesbina* Shaw. Z Physiol Chem 1939;262:53–60.

Kurosawa T, Nakano H, Sato M, Tohma M. Synthesis of 3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-5β-cholestan-26-oic acids by the use of β-ketosulfoxide. Steroids 1995;60:439–444. [PubMed: 7482627]

Lavoué S, Miya M, Poulsen JY, Møller PR, Nishida M. Monophyly, phylogenetic position and inter-familial relationships of the Alepocephaliformes (Teleostei) based on whole mitogenome sequences. Mol Phylogenet Evol 2008;47:1111–1121. [PubMed: 18262798]

Lloyd MD, Darley DJ, Wierzbicki AS, Thrreadgill MD. α-Methylacyl-CoA racemase – an 'obscure' metabolic enzyme takes centre stage. FEBS J 2008;275:1089–1102. [PubMed: 18279392]

Mabuti H. Uber Trioxy-bis-nor-sterocholansaure $C_{26}H_{44}O_5$ aus der Galle von *Rana catesbina* Shaw. J Biochem 1941;33:117–130.

Matsumoto Y, Oota H, Asaoka Y, Nishina H, Watanabe K, Bujnicki JM, Oda S, Kawamura S, Mitani H. Medaka: a promising model animal for comparative population genomics. BMC Res Notes 2009;2:88. [PubMed: 19426554]

Nagane Gowda GA, Ijare OB, Somashekar BS, Sharma A, Kapoor VK, Khetrapal CL. Single-step analysis of individual conjugated bile acids in human bile using ^{1}H NMR spectroscopy. Lipids 2006;41:591–603. [PubMed: 16981437]

Nelson, JS. Fishes of the World. Hoboken, NJ: John Wiley and Sons, Inc.; 2006.

Parenti LR. A phylogenetic analysis and taxonomic revision of ricefishes, *Oryzias* and relatives (Beloniformes, Adrianichthyidae). Zool J Linnean Soc 2008;154:494–610.

Reschly EJ, Ai N, Ekins S, Welsh WJ, Hagey LR, Hofmann AF, Krasowski MD. Evolution of the Bile Salt Nuclear Receptor FXR in Vertebrates. J Lipid Res 2008;49:1577–1587. [PubMed: 18362391]

Shefer S, Cheng FW, Batta AK, Dayal B, Tint S, Salen G, Mosbach EH. Stereospecific side chain hydroxylations in the biosynthesis of chenodeoxycholic acid. J Biol Chem 1978;253:6386–6392. [PubMed: 28327]

Shinoda, K.; Nakagawa, T.; Tomamushi, B.; Isemura, T. Colloidal Surfactants. New York: Academic Press Inc; 1963.

Starchenkov I, Trapencieris P, Kauss V, Jas G, Kalvinsh I. A convenient synthesis of 5β-cholestan-26-oic and 5β-cholestan-26,27-dioic acids. Steroids 2000;65:143–147. [PubMed: 10699593]

Une M, Hoshita T. Natural occurrence and chemical synthesis of bile alcohols, higher bile acids, and short side chain bile acids. Hiroshima J Med Sci 1994;43:37–67. [PubMed: 7928396]

Une M, Morigami I, Kihira K, Yasuhara M, Kuramoto T, Hoshita T. Stereospecific formation of (24*E*)-3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid and (24*R*,25*S*)-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid from either (25*R*)- or (25*S*)-3α,7α,12α-trihydroxy-5β-cholestan-26 oic acid by rat liver homogenate. J Biochem 1984;96:1103–1107. [PubMed: 6520115]

Une M, Nagai F, Hoshita T. Comparative biochemical studies of bile acids and bile alcohols. High-performance liquid chromatographic separation of higher bile acids. J. Chromatogr 1983;257:411–415. [PubMed: 6602140]

Une M, Tazawa Y, Tada K, Hoshita T. Occurrence of both (25*R*)-and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestanoic acids in urine from an infant with Zellweger's syndrome. J Biochem 1987;102:1525–1530. [PubMed: 3448094]

Van Veldhoven PP, Croes K, Asselberghs S, Herdewijn P, Mannaerts GP. Peroxisomal β-oxidation of 2-methyl branched acyl-CoA esters: stereospecific recognition of the 2*S*–methyl compounds by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase. FEBS Lett 1996;388:80–84. [PubMed: 8654595]

Fig. 1.
Photograph of the Japanese rice fish, the medaka (*Oryzias latipes*).

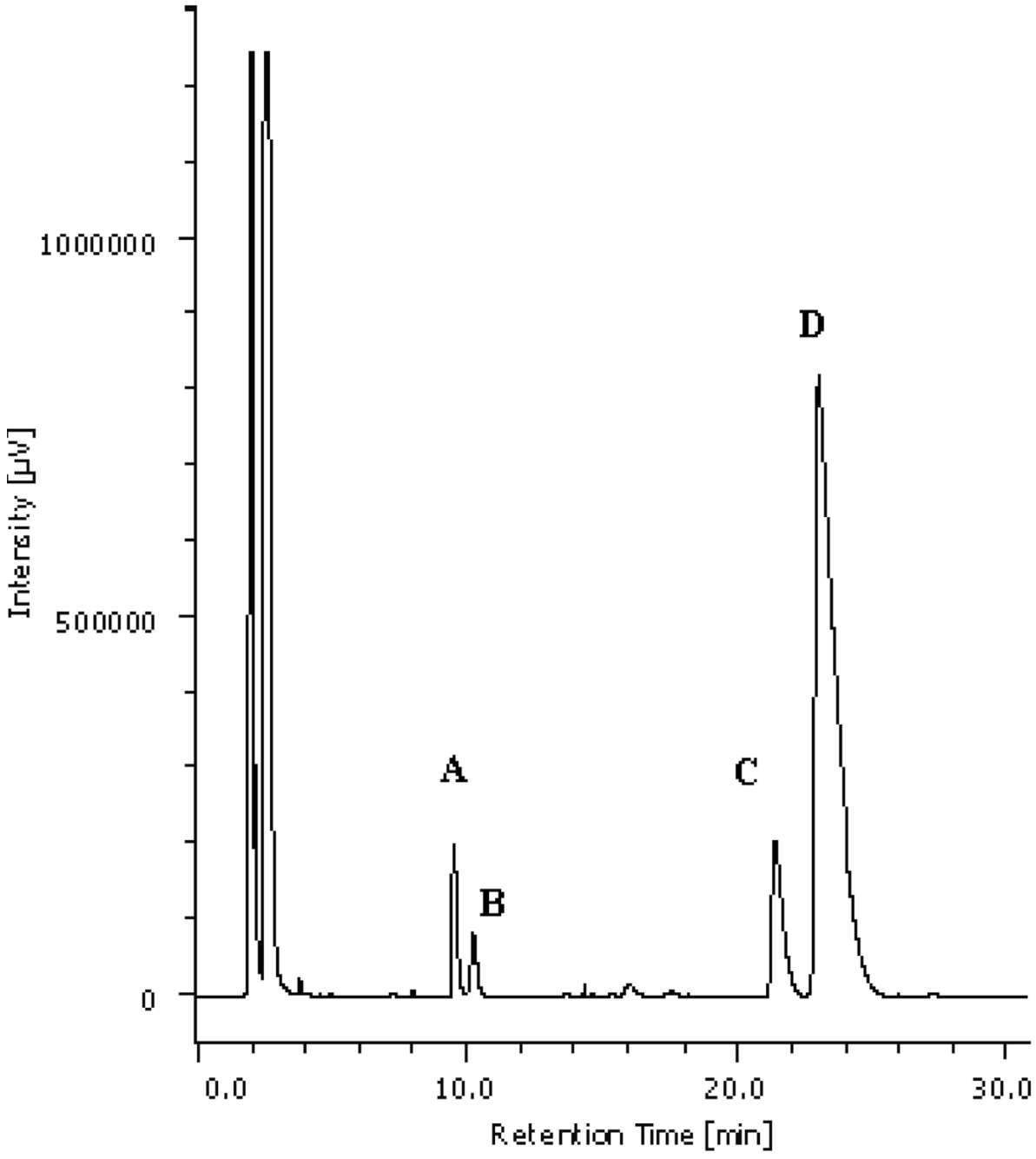

Fig. 2.

RP-HPLC (with an ELSD) profile of the bile acids of the medaka.

Peak A, 5% of biliary bile acids, was identified as cholyl taurine; its retention time (RT) was 9.6 min; peak B, 2%, was not identified, RT 10.3 min; Peak C, 11%, was identified as (25*S*)-3α,7α,12α-trihydroxy-5β-cholestan-27-oyl taurine, RT 21.3 min; and peak D, 82%, was identified as (25*R*)-3α,7α,12α-trihydroxy-5β-cholestan-27-oyl taurine, RT 23.1 min.

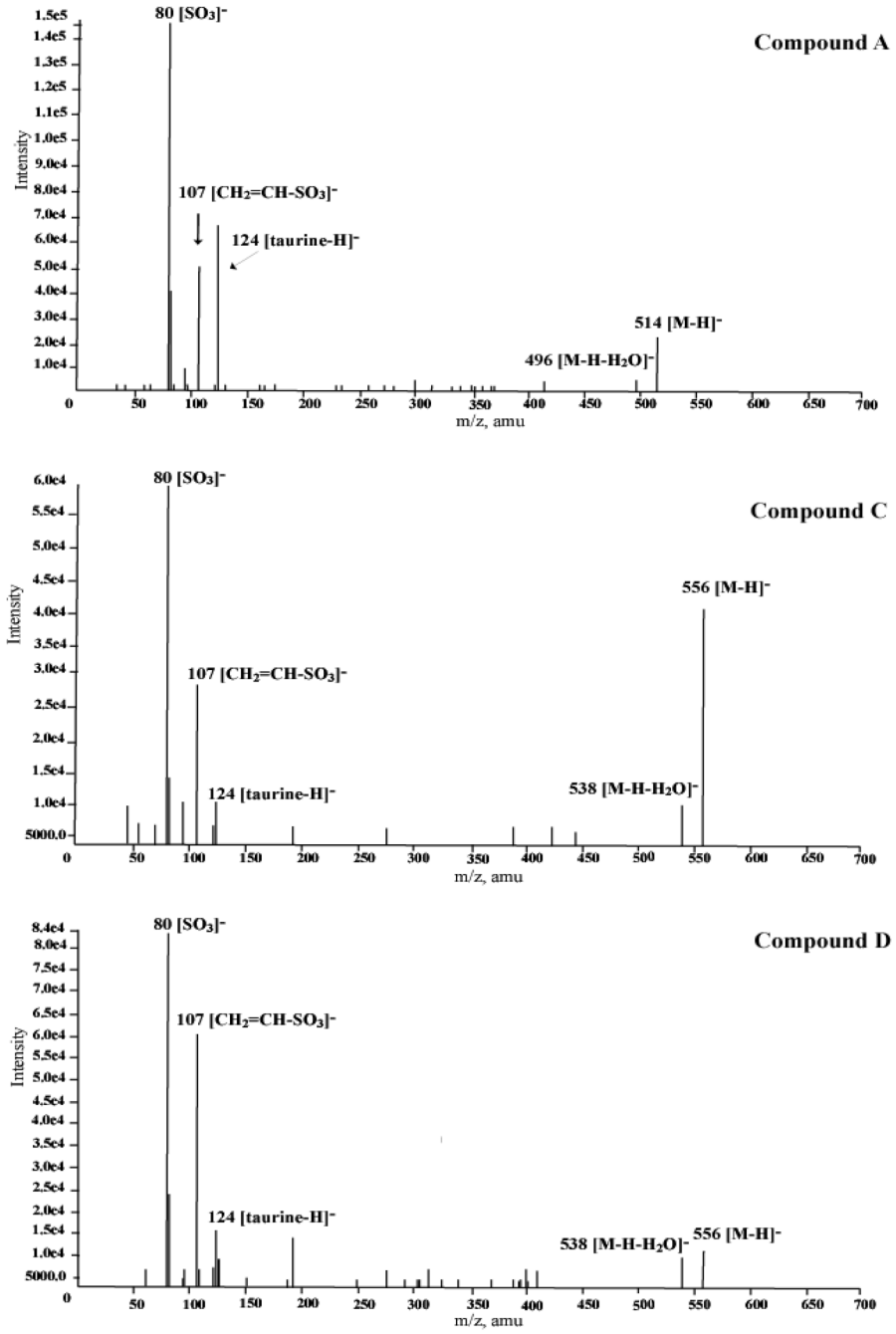

Fig. 3.
LC/ESI-MS/MS spectra of the isolated compounds A, C, and D.

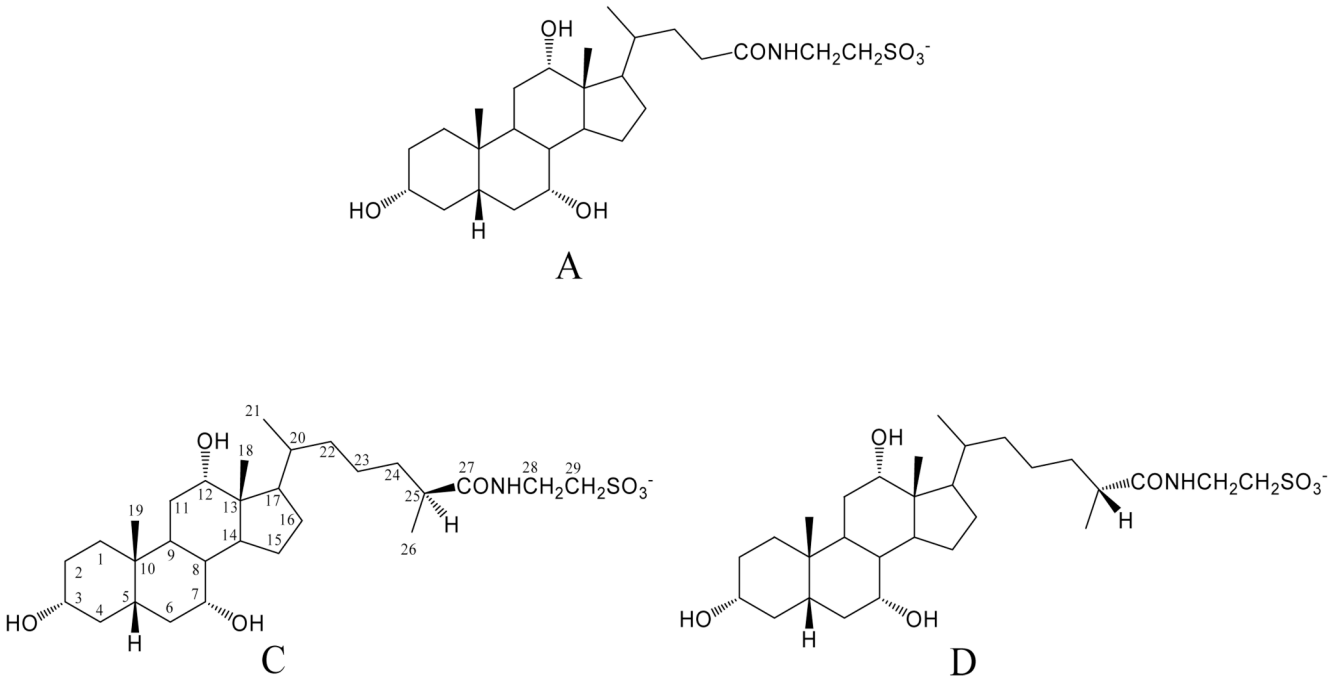

Fig. 4.

Structures of the C_{24} and C_{27} trihydroxy bile acids isolated from the medaka: cholyl taurine (compound A); (25*S*)-3α,7α,12α- trihydroxy-5β-cholestan-27-oyl taurine (compound C); and (25*R*)- 3α,7α,12α-trihydroxy-5β-cholestan-27-oyl taurine (compound D).

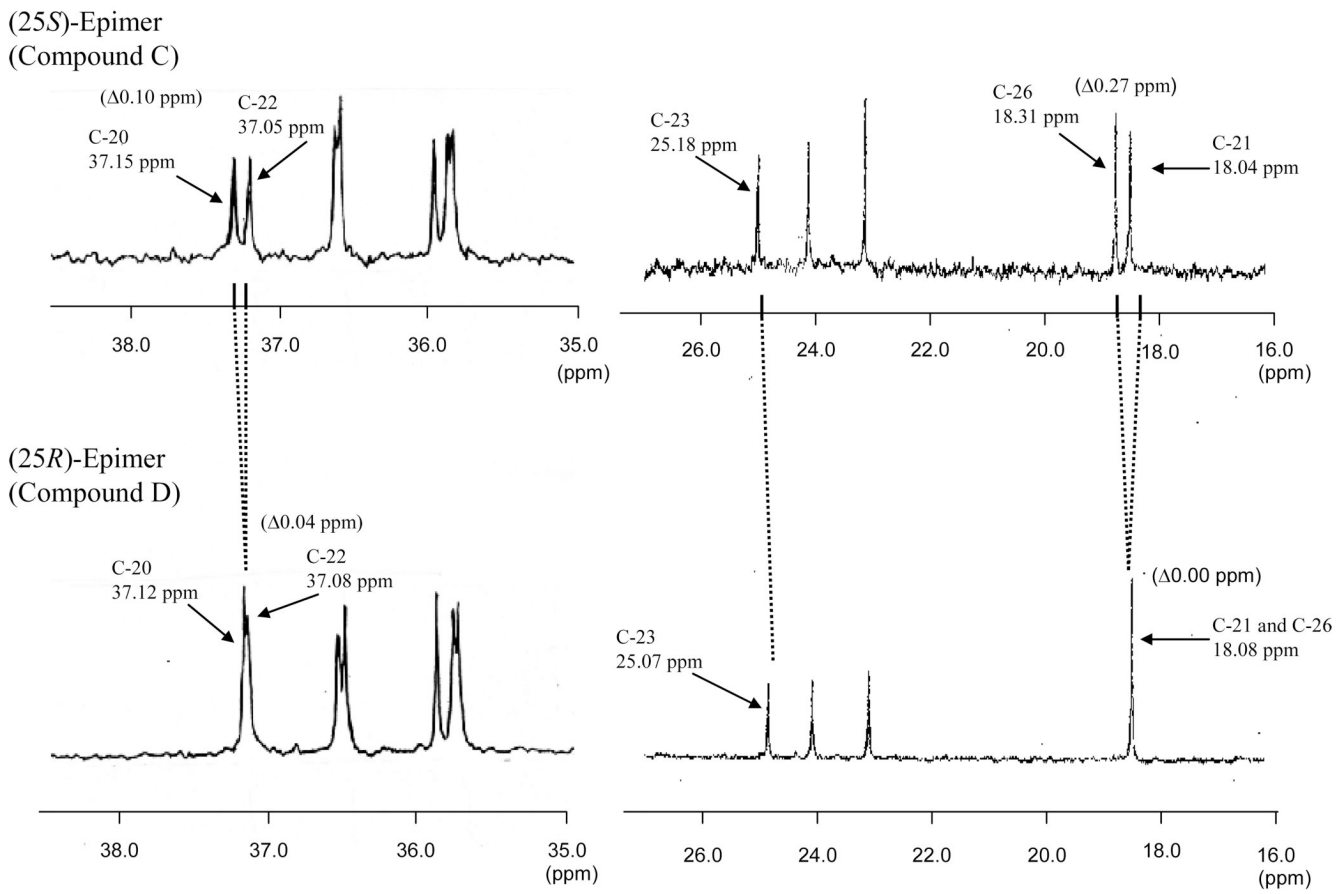

Fig. 5.
Partial ^{13}C NMR spectra of the isolated compounds C and D.

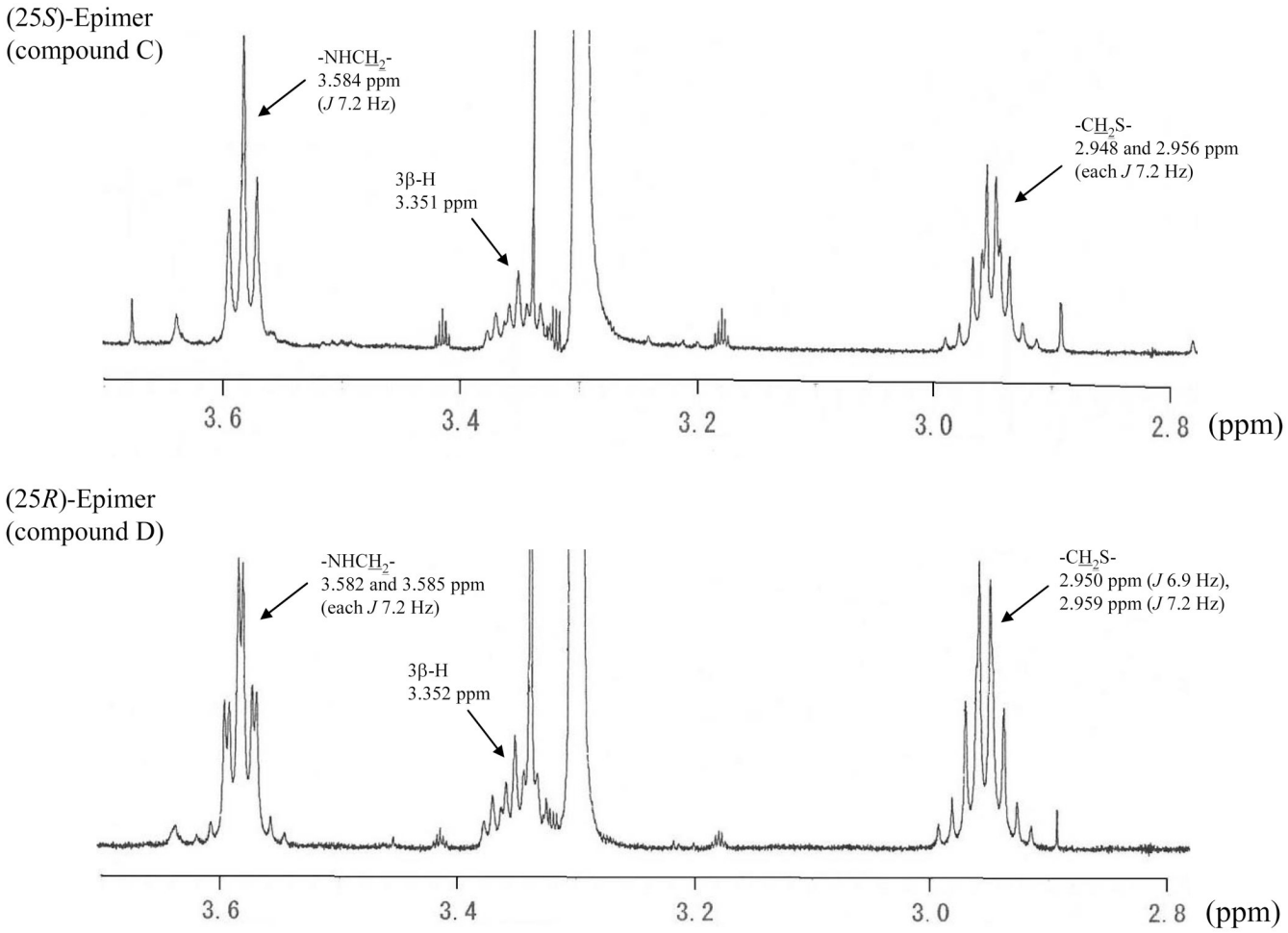

Fig. 6.
Partial ^{1}H NMR spectra of the isolated compounds C and D.

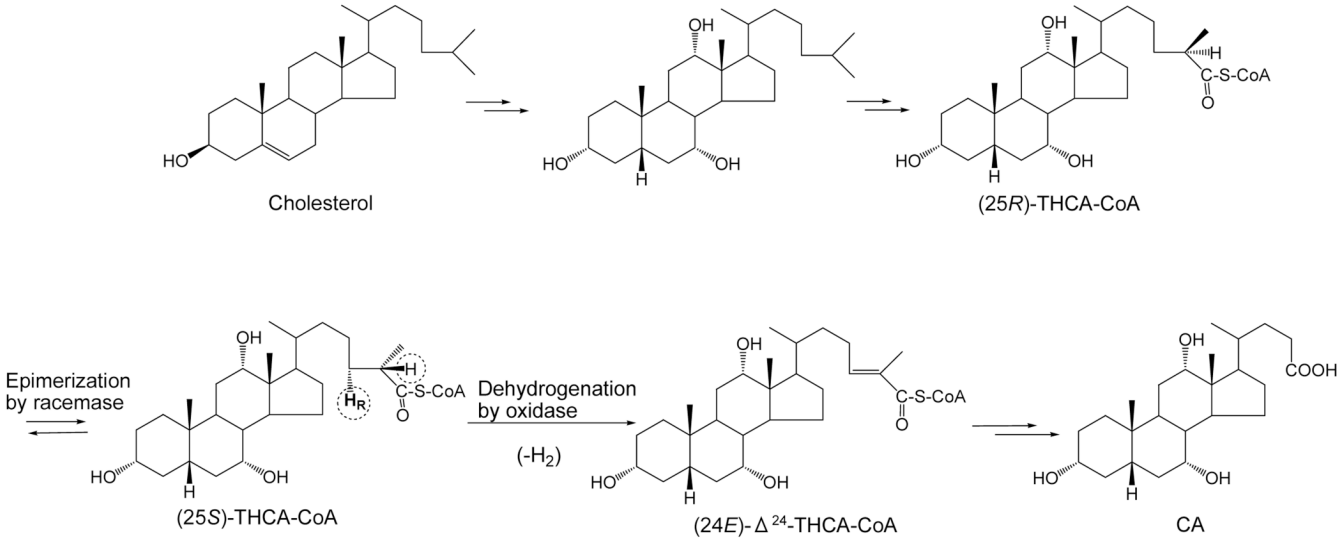

Fig. 7.

Early steps in the “neutral” biosynthetic pathway of CA from cholesterol in vertebrates. The two epimers of the CoA thioester of THCA are shown.

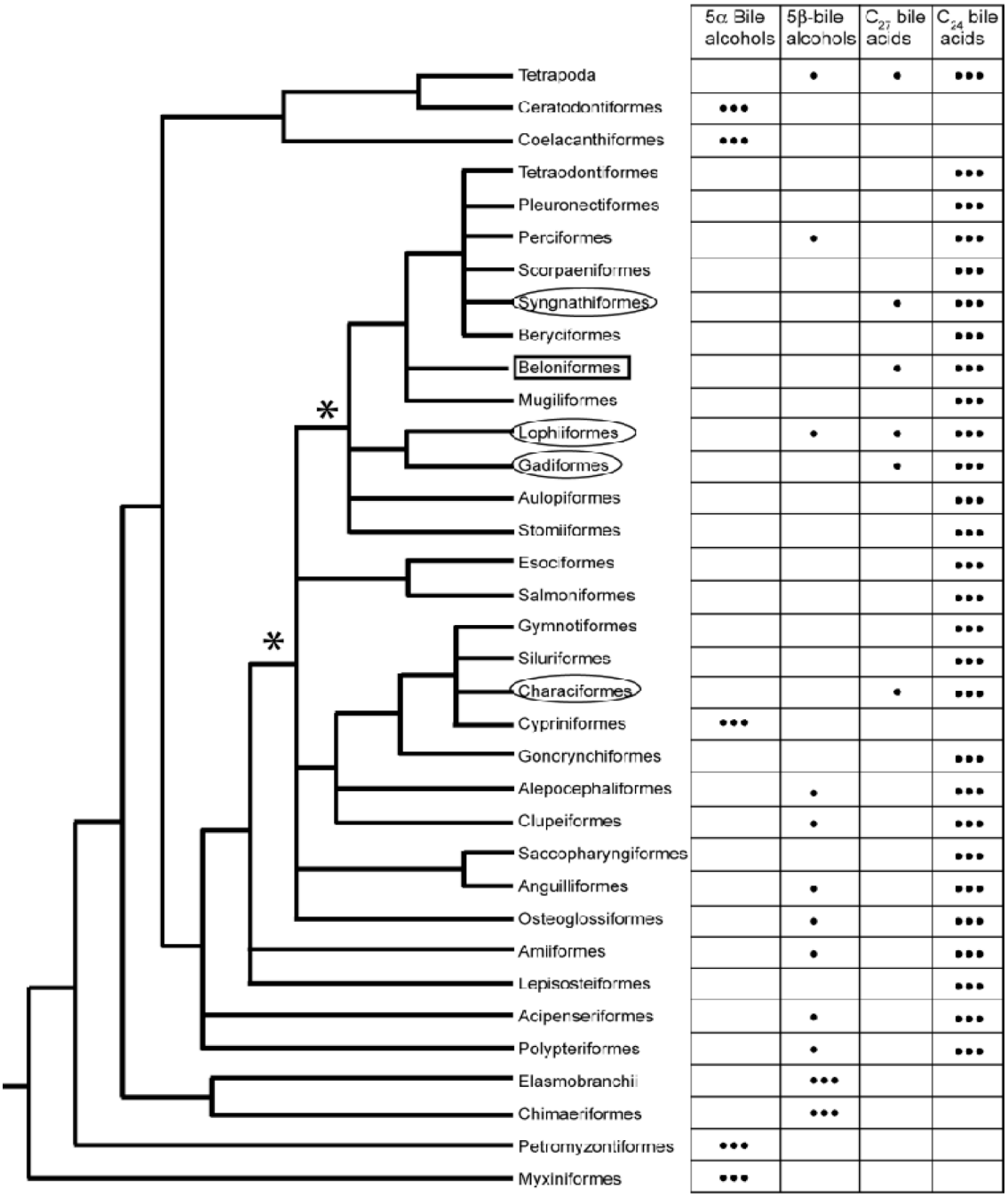

Fig. 8.

Bile salt species variation determined by Hagey et al. (2010) overlaid on the fish tree proposed by Nelson (2006) excluding fish orders whose bile salt profiles are unknown. Alepocephaliformes is used instead of Argentiformes following recent results of Lavoué et al. (2008), and Syngnathiformes is used instead of Gasterosteiformes following the results of Kawahara et al. (2008). The major bile salts for each species are indicated by ●●●; minor bile salts are indicated by ●. Medaka is classified in Beloniformes (box). Other than Beloniformes, only four other fish orders (circled) have species which have C_{27} bile acids comprising greater than 10% of total biliary bile salts. C_{27} bile acids are not found as major components of biliary bile salts in jawless or cartilaginous fish, or in basal teleost fish (e.g., Polypteriformes, bichirs;

Acipenseriformes, including paddlefishes and sturgeons). Ancestral fish that may have used C_{27} bile acids are indicated by the * at two nodes in the phylogenetic tree.

Table 1

Complete ^{1}H and ^{13}C chemical shifts of the isolated taurine conjugates of (25*R*)/ (25*S*)-THCAs.[a]

	Compound C (25*S*)				Compound D (25*R*)				Difference in the chemical shifts between C and D		
Carbon no.	Type	^{13}C	^{1}H		Type	^{13}C	^{1}H		^{13}C	^{1}H	
			α	β			α	β		α	β
1	CH_2	36.52	1.80	0.96	CH_2	36.52	1.79	0.96	0.00	0.01	0.00
2	CH_2	31.21	1.42	1.58	CH_2	31.20	1.41	1.58	0.01	0.01	0.00
3	CH	72.93		3.35 (brm)	CH	72.92		3.35 (brm)	0.01	0.00	
4	CH_2	40.50	2.26	1.64	CH_2	40.50	2.26	1.64	0.00	0.00	0.00
5	CH	43.25		1.36	CH	43.24		1.36	0.01	0.00	
6	CH_2	35.83[b]	1.50	1.94	CH_2	35.81[b]	1.50	1.94	0.02	0.00	0.00
7	CH	69.10		3.78 (m)	CH	69.11		3.78 (m)	−0.01	0.00	
8	CH	41.07		1.54	CH	41.08		1.54	−0.01	0.00	
9	CH	27.89	2.24		CH	27.87	2.24		0.02	0.00	
10	C	35.92			C	35.92			0.00		
11	CH_2	29.57	1.56	1.56	CH_2	29.57	1.56	1.56	0.00	0.00	0.00
12	CH	74.05		3.94 (m)	CH	74.09		3.94 (m)	−0.04	0.00	
13	C	47.47			C	47.47			0.00		
14	CH	42.97	1.96		CH	42.95	1.95		0.02	0.01	0.00
15	CH_2	24.24	1.72	1.09	CH_2	24.22	1.73	1.09	0.02	−0.01	0.00
16	CH_2	28.86	1.86	1.26	CH_2	28.82	1.86	1.26	0.04	0.00	0.00
17	CH	48.34	1.84		CH	48.30	1.82		0.04	0.02	
18	CH_3	12.99	0.70 (s)		CH_3	13.00	0.70 (s)		−0.01	0.00	
19	CH_3	23.16	0.91 (s)		CH_3	23.16	0.91 (s)		0.00	0.00	
20	CH	37.15	1.38		CH	37.12	1.38		0.03	0.00	
21	CH_3	18.04	0.98 (d, 6.6)		CH_3	18.08	0.98 (d, 6.6)		−0.04	0.00	
22	CH_2	37.05	1.06, 1.38		CH_2	37.08	1.04, 1.38		−0.03	0.02, 0.00	
23	CH_2	25.18	1.38		CH_2	25.07	1.20, 1.34		0.11	0.04	
24	CH_2	35.80[b]	1.26, 1.52		CH_2	35.78[b]	1.34, 1.52		0.02	−0.08, 0.00	

	Compound C (25*S*)				Compound D (25*R*)				Difference in the chemical shifts between C and D		
Carbon no.	**Type**	**^{13}C**	**^{1}H**		**Type**	**^{13}C**	**^{1}H**		**^{13}C**	**^{1}H**	
			α	**β**			**α**	**β**		**α**	**β**
25	CH	42.33	2.24		CH	42.35	2.26		−0.02	−0.02	
26	CH_3	18.31	1.08 (d, 6.6)		CH_3	18.08	1.08 (d, 6.6)		0.23	0.00	
27	C	179.40			C	179.50			−0.10		
28	CH_2	36.49	3.58 (t, 7.2)		CH_2	36.48	3.58, 3.59 (each t, 7.2)		0.01	0.00, −0.01	
29	CH_2	51.58	2.95, 2.96 (each t, 7.2)		CH_2	51.59	2.95 (t, 6.9), 2.96 (t, 7.2)		−0.01	0.00, 0.00	

[a] Measured in CD_3OD at 600 MHz in ^{1}H-NMR and at 149.4 MHz in ^{13}C-NMR; chemical shifts were expressed as δ ppm relative to Me_4Si; abbreviations used: s, singlet; d, doublet; m, multiplet; brm, broad multiplet; t, triplet; values in parentheses refer to signal multiplicity and coupling constant (*J* in Hz).

[b] Assignment down a vertical column may be interchanged.