

Physicochemical and physiological properties of 5 α -cyprinol sulfate, the toxic bile salt of cyprinid fish

T. Goto,^{1,*} F. Holzinger,^{2,*} L. R. Hagey,^{*} C. Cerrè,^{3,*} H-T. Ton-Nu,^{4,*} C. D. Scheingart,^{5,*} J. H. Steinbach,^{*} B. L. Shneider,[†] and A. F. Hofmann^{6,*}

Division of Gastroenterology,^{*} Department of Medicine, University of California, San Diego, CA 92093-0813; and the Department of Pediatrics,[†] Mount Sinai School of Medicine, New York, NY 10029-0313

Abstract 5 α -Cyprinol sulfate was isolated from bile of the Asiatic carp, *Cyprinus carpio*. 5 α -Cyprinol sulfate was surface active and formed micelles; its critical micellization concentration (CMC) in 0.15 M Na⁺ using the maximum bubble pressure device was 1.5 mM; by dye solubilization, its CMC was \sim 4 mM. At concentrations >1 mM, 5 α -cyprinol sulfate solubilized monooleylglycerol efficiently (2.1 molecules per mol micellar bile salt). When infused intravenously into the anesthetized rat, 5 α -cyprinol sulfate was hemolytic, cholestatic, and toxic. In the isolated rat liver, it underwent little biotransformation and was poorly transported ($T_{\max} \cong 0.5 \mu\text{mol}/\text{min}/\text{kg}$) as compared with taurocholate. 5 α -Cyprinol, its bile alcohol moiety, was oxidized to its corresponding C₂₇ bile acid and to alcoholic acid (the latter was then conjugated with taurine); these metabolites were efficiently transported. 5 α -Cyprinol sulfate inhibited taurocholate uptake in COS-7 cells transfected with rat *asbt*, the apical bile salt transporter of the ileal enterocyte. 5 α -Cyprinol had limited aqueous solubility (0.3 mM) and was poorly absorbed from the perfused rat jejunum or ileum. Sampling of carp intestinal content indicated that 5 α -cyprinol sulfate was present at micellar concentrations, and that it did not undergo hydrolysis during intestinal transit. These studies indicate that 5 α -cyprinol sulfate is an excellent digestive detergent and suggest that a micellar phase is present during digestion in cyprinid fish.—Goto, T., F. Holzinger, L. R. Hagey, C. Cerrè, H-T. Ton-Nu, C. D. Scheingart, J. H. Steinbach, B. L. Shneider, and A. F. Hofmann. Physicochemical and physiological properties of 5 α -cyprinol sulfate, the toxic bile salt of cyprinid fish. *J. Lipid Res.* 2003. 44: 1643–1651.

Supplementary key words *Cyprinus carpio* • bile acids • micelles • bacterial deconjugation • fat digestion • fat absorption • hepatic transport • cholestasis • intestinal absorption • solubilization

In vertebrates, cholesterol is eliminated by conversion to water-soluble amphipathic, functional molecules called bile salts. Bile salts can be divided into three classes based on side-chain structure: C₂₇ bile alcohols, C₂₇ bile acids, and C₂₄ bile acids (1). After their biosynthesis from cholesterol, bile alcohols and bile acids undergo “conjugation,”

a biotransformation step that renders them water soluble and membrane impermeable at physiological pH. Bile alcohols are conjugated by esterification of the terminal C-27 hydroxy group with sulfate, whereas bile acids are usually conjugated by N-acyl amidation of the terminal C-27 or C-24 carboxyl group with taurine or glycine (2, 3).

The occurrence of C₂₇ bile alcohol sulfates is widespread in nature. They are the dominant bile salts of ancient mammalian species (elephant, manatee, hyrax, and rhinoceros) (4). They are also the major biliary surfactants present in cartilaginous fish (sharks, rays, and skates), herbivorous bony fish (carp, arapima, and angelfish), and in some amphibians (salamanders and frogs) (3, 5).

One of the common bile alcohols is 5 α -cyprinol, a molecule with five hydroxy groups that was originally isolated from the bile of *Cyprinus carpio*, the Asiatic carp. Cyprinol was shown to have hydroxy groups at C-3, C-7, C-12, C-26, and C-27, based on the work of Hoshita, Magayoshi, and Kazuno (6) and Anderson, Briggs, and Haslewood (7). Confirmation of the structure of the sulfate ester of 5 α -cyprinol by proton and ¹³C-NMR as well as mass spectrometry (MS) has been reported by Asakawa et al. (8)⁷. The A/B ring juncture of cyprinol is 5 α (A/B *trans*), whereas the structure of most C₂₇ and C₂₄ bile acids is 5 β (A/B *cis*). It has become customary to add a 5 α prefix to cyprinol to indicate clearly its 5 α -A/B *trans* juncture, and thus distinguish it from 5 β -cyprinol (A/B *cis*), which is present in other fish, such as the sturgeon (9). The structure of 5 α -cyprinol sulfate is shown in Fig. 1.

¹ Present address for T. Goto: Department of Chemistry and Biochemistry, Numazu College of Technology, Numazu, Shizuoka 410-8501, Japan.

² Present address for F. Holzinger: Department of Surgery, Inselspital, University of Bern, Switzerland.

³ Present address for C. Cerrè: Bioikos Farma s.r.l, 40122 Bologna, Italy.

⁴ Present address for H-T. Ton-Nu: La Jolla Pharmaceutical Company, San Diego, CA 92121.

⁵ Present address for C. D. Scheingart: Ferring Research Institute, San Diego, CA 92121.

⁶ To whom correspondence should be addressed.
e-mail: ahofmann@ucsd.edu

⁷ Chemical Abstracts has assigned the registry number 15066-41-8 to 5 α -cyprinol sulfate. Its index name is Cholestane-3,7,12,26,27-pentol, hydrogen sulfate, (3 α ,5 α ,7 α ,12 α)⁻. The assignment of the sulfate to C-27 versus C-26 is arbitrary.

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Most natural bile acids are amphipathic, possessing a hydrophilic side and a hydrophobic side (10). The amphipathic structure of bile acids is responsible for their chief physiological function, which is to enhance absorption of dietary lipids. The C₂₄ bile acids readily form mixed micelles with fatty acids and monoglycerides, and such mixed micelles can in turn solubilize fat-soluble vitamins. Such solubilization greatly enhances diffusion of insoluble lipids to the enterocyte brush border (11).

The physicochemical properties of C₂₄ bile acids have been investigated extensively (12, 13), but few studies have examined the physicochemical properties of C₂₇ bile acids and C₂₇ bile alcohols. We hypothesized that the micelle-forming and solubilization properties of 5 α -cyprinol sulfate should be similar to those of taurocholate, a molecule with a similar topology, as shown in Fig. 2, and performed studies to test this hypothesis. We also performed limited physiological studies on its ileal and hepatic transport in rodents because of its known toxicity for mammals (14–17), including humans [reviewed in (17)]. Finally, we examined some properties of 5 α -cyprinol, the bile alcohol moiety of 5 α -cyprinol sulfate, in order to define the possible *in vivo* significance of bacterial hydrolysis (deconjugation) of the ester bond linking sulfate to the bile alcohol.

METHODS

Isolation of 5 α -cyprinol sulfate from carp bile

Gallbladders of *C. carpio* were obtained from a local fish market and an aquaculture facility (Loy Fisheries, Provo, UT) and stored in isopropanol. 5 α -Cyprinol sulfate was isolated from the isopropanol-soluble extract of carp gallbladders. The extract was subjected to flash chromatography using a 30 \times 5 cm column packed to 21 cm with silica gel, 40 μ m (Flash Chrom Pack, J. T. Baker, Phillipsburg, NJ). The column was packed in chloroform-methanol (80:20; v/v). A highly concentrated isopropanol extract of carp bile was layered at the top of the column. A stepwise gradient of methanol in chloroform (80:20, 500 ml; 75:25, 500 ml; 70:30, 1,000 ml; 65:35, 500 ml) was used to elute the 5 α -cyprinol sulfate. Fractions were examined by thin-layer chromatography (TLC) using a solvent system for conjugated bile acids (18). Fractions containing pure 5 α -cyprinol sulfate (*R_f* 0.25) were pooled and taken to dryness on a rotary evaporator.

The structure of 5 α -cyprinol sulfate (5 α -cholestane-3 α ,7 α ,12 α ,26,27-pentol-27-sulfate) was confirmed by proton magnetic resonance spectroscopy. Proton ¹H-NMR was carried out at 500 MHz in the Department of Chemistry, University of California, San Diego. The solvent was deuterated methanol, and chemical shifts are expressed in ppm relative to tetramethylsilane: 0.697 (s, 3H, Me-18), 0.793 (s, 3H, Me-19), 0.996 (d, 7.0 Hz, 3H, Me-21), 2.129 (tt, 12.5 Hz, 3.5 Hz, 1H, H-5), 3.540 ν and 3.566 ν (ABX, *J_{ab}* 11.0 Hz, *J_{ax}* 6.6 Hz, *J_{bx}* 5.6 Hz, 2H, H-27), 3.765 (d, 5.0 Hz, 1H, H-7), 3.928 (m, 1H, H-3), 3.960 (s, 1H, H-12), 3.985 ν and 4.015 ν (ABX, *J_{ab}* 9.5 Hz, *J_{ax}* 5.0 Hz, *J_{bx}* 6.5 Hz, 2H, H-26).

Preparation of 5 α -cyprinol by solvolysis of 5 α -cyprinol sulfate

5 α -Cyprinol sulfate was precipitated from the isopropanol extract of carp gallbladders by the addition of several volumes of ethyl acetate. The precipitate (1.4 g) was dissolved in 2,2'-dimethoxypropane-1 N HCl (7:1; v/v) and maintained at 37°C for 12 h (19), the

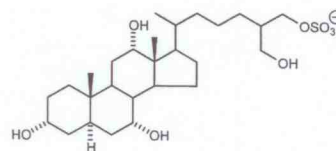


Fig. 1. Chemical structure of 5 α -cyprinol sulfate.

procedure resulting in complete solvolysis of the 5 α -cyprinol sulfate. Water (200 ml) and chloroform-methanol (2:1, v/v) (800 ml) were added. The chloroform phase was evaporated to dryness, giving impure 5 α -cyprinol. This was purified by silica gel column chromatography using chloroform-methanol, with stepwise increases in the proportion of methanol. Fractions were examined by TLC (18), and those containing pure 5 α -cyprinol (*R_f* 0.66) were pooled to give 0.9 g of 5 α -cyprinol that was pure by TLC. The molecular weight of 5 α -cyprinol was confirmed by electrospray (ESI)-MS.

Physicochemical properties of 5 α -cyprinol sulfate and 5 α -cyprinol

Determination of critical micellization temperature of 5 α -cyprinol sulfate. The critical micellization temperature (CMT) (also termed Krafft point) is the temperature at which the solubility of the monomer reaches the critical micellization concentration (CMC). At this temperature, there is a phase change: insoluble, crystalline material dissolves and forms micelles. A 20 mM solution of 5 α -cyprinol sulfate in water was kept at 4°C and observed daily for 4 days to see if 5 α -cyprinol sulfate precipitated from solution.

Determination of ion product of the calcium salt of 5 α -cyprinol sulfate. Bottles were prepared containing three bile salt concen-

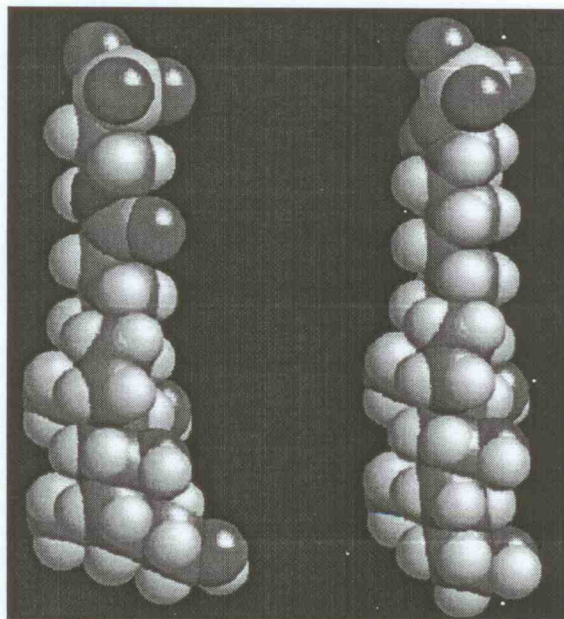


Fig. 2. Space-filling models of taurocholate (cholytaurine), left panel, and 5 α -cyprinol sulfate, right panel. Each molecule has a hydrophilic side and a hydrophobic side, and they are thus similar planar amphipaths. The *cis* A/B structure of taurocholate can be seen (bottom), as well as the nitrogen atom of taurine. The two molecules are quite similar in topology.

