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Comparative Studies of Bile Salts

5α-CHIMAEROL, A NEW BILE ALCOHOL FROM THE WHITE SUCKER *CATOSTOMUS COMMERSONI* LACÉPÈDE

BY I. G. ANDERSON AND G. A. D. HASLEWOOD
*Guy's Hospital Medical School, London S.E.*1, *U.K.*

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1. G.l.c. examination of bile alcohols prepared from the sucker *Catostomus commersoni* Lacépède (family Catostomidae) showed that although 5α-cyprinol (5α-cholestane-3α,7α,12α,26,27-pentol) was a minor constituent, the principal bile alcohol was an undescribed substance, probably present in the bile as the C-26 sulphate ester, whose i.r., n.m.r. and mass spectra agreed with the structure 5α-cholestane-3α,7α,12α,24,26-pentol. 2. M_D studies suggest that this *5α-chimaerol* is the 24(+), 25*S* enantiomer and that 5β-chimaerol (chimaerol) from *Chimaera monstrosa* bile also has the 24(+), 25*S* configuration. These findings imply that bile alcohol biosynthesis in suckers and chimaeras includes stereospecific oxidation of cholesterol at C-26. 3. *C. commersoni* bile acids (present in minor amounts) probably consist largely of 3α,7α,12α-trihydroxy-5α-cholan-24-oic acid (allocholic acid). 4. 5α-Chimaerol sulphate and 5α-cyprinol sulphate are probably biochemically equivalent as bile salts, and can be considered as arising by parallel evolution.

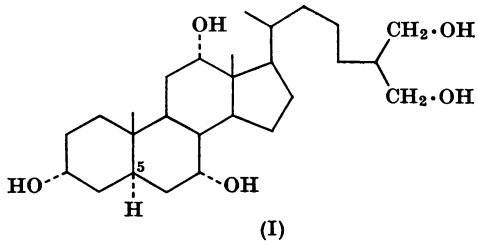
A bile alcohol, cyprinol (5α-cyprinol), was found as the monosulphate in the common carp *Cyprinus carpio* L. (Haslewood, 1955) and has since been isolated from at least eleven other species of the family Cyprinidae and probably also from a loach of the family Cobitidae (references in Haslewood, 1967). All these fish are placed in the huge mainly freshwater order Ostariophysi; the presence of 5α-cyprinol has been regarded as a primitive character suggesting that the Cyprinidae and Cobitidae families are evolutionarily less advanced than other ostariophysians so far investigated. Since the cyprinids have a very long, perhaps exclusively, freshwater history, 5α-cyprinol has also been regarded as a characteristically freshwater type of bile alcohol; for example, its presence in coelacanth (*Latimeria chalumnae* Smith) bile is consistent with the freshwater history of these forms (Haslewood, 1964).

Bile from the North American white sucker *Catostomus commersoni* Lacépède had a strong superficial resemblance to that of carp, and the chemical, spectroscopic and chromatographic behaviour of the principal bile salt suggested that it was 5α-cyprinol sulphate (Haslewood, 1967), an acceptable suggestion since the suckers, family Catostomidae, are closely related to the Cyprinidae. However, g.l.c. showed that the principal *Catostomus* bile alcohol is not 5α-cyprinol: its chemistry is described below.

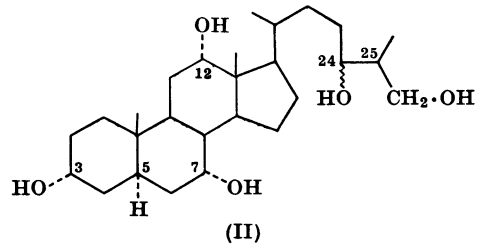
RESULTS

Paper chromatography of *C. commersoni* bile salts gave a principal spot with the mobility of 5α-cyprinol sulphate and a lesser spot that moved at the same rate as taurocholate, a pattern shown by all the cyprinid bile salts so far examined. Dioxan–trichloroacetic acid cleavage gave a neutral product, m.p. 235°C, $[\alpha]_D$ +33.5°, which had an i.r. spectrum closely similar to that of 5α-cyprinol [5α-cholestane-3α,7α,12α,26,27-pentol (I)] and almost the same mobility as that compound on paper and thin-layer chromatography. G.l.c. of the TMS* ethers showed that the principal constituent had a shorter retention time than TMS-5α-cyprinol although a shoulder on the recorded tracing indicated that a very small proportion of the latter substance might also be present. The findings reminded us of the close resemblance between 5β-cyprinol (5β-cholestane-3α,7α,12α,26,27-pentol) and chimaerol ('5β-chimaerol', 5β,25ξ-cholestane-3α,7α,12α,24ξ,26-pentol) previously discussed (Bridgwater, Briggs & Haslewood, 1962; Haslewood & Tammar, 1968) and we suggested to Dr L. Tökés that the new alcohol, characterized as a tetra-acetate, might be '5α-chimaerol' [5α,25ξ-cholestane-3α,7α,12α,24ξ,26-pentol (II)]; his n.m.r. and mass spectral analyses (Appendix to this paper) confirm this suggestion except for the location of the C-24 hydroxyl group.

*Abbreviation: TMS, trimethylsilyl.



(I)



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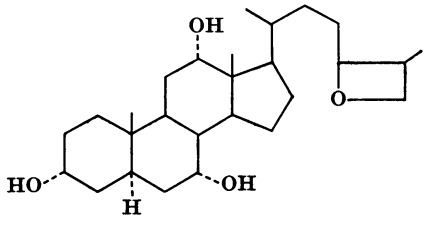

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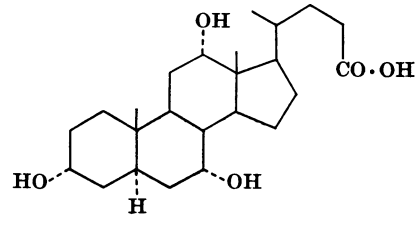
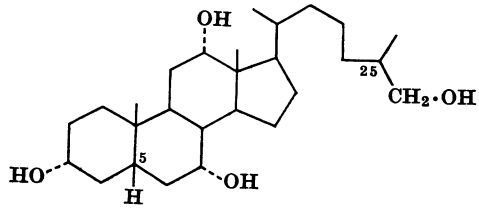


(IV)



(V)

Alkaline hydrolysis of *C. commersoni* bile salts gave, as almost the sole neutral product, a crystalline compound m.p. 222°C, $[\alpha]_D$ +32°, regarded as anhydro 5α-chimaerol (III), which closely resembled anhydro 5α-cyprinol in its i.r. spectrum (Haslewood, 1967) and mobility on t.l.c. but had, as its TMS ether, a shorter retention time on g.l.c. The broad band in the i.r. spectrum of anhydro 5α-chimaerol with a maximum at about 10.4μm and characteristic of the trimethylene oxide ring (Cross, 1960) establishes, together with observations by Tökés (Appendix to this paper), that a hydroxyl group must be located at C-24 in 5α-chimaerol itself.

Crude crystalline anhydro 5α-chimaerol showed some minor spots on t.l.c., one corresponding in mobility to 5α-chimaerol, and on g.l.c. the TMS ether gave a definite secondary peak with the retention time of anhydro TMS-5α-cyprinol. This is fairly convincing evidence that *C. commersoni* bile salts also contain minor amounts of the sulphate of 5α-cyprinol.

The small (about 0.2% by weight) acidic fraction of hydrolysed *C. commersoni* bile salts, after methylation and conversion into TMS ethers, gave a sharp peak on g.l.c. with the retention time of similarly treated allocholic acid [3α,7α,12α-trihydroxy-5α-cholan-24-oic acid (IV)] and a second smaller broad peak, the maximum retention time of which differed somewhat from that of methyl TMS-cholate (methyl TMS-3α,7α,12α-trihydroxy-5β-cholan-24-oate). This evidence does not show that cholic acid is not present, but strongly suggests that the principal bile acid is its 5α-epimer (IV).

The mobility of the principal bile salt on chromatograms and the quantity of SO_4^{2-} found on hydrolysis strongly suggest that 5α-chimaerol occurs in the bile salts as its monosulphate ester. Attempts to locate the position of the ester group by chromic oxidation and acid hydrolysis as in previous work (e.g. Haslewood & Tökés, 1969) gave a small yield of a complex mixture of unidentified acids. However, the i.r. spectrum of *C. commersoni* bile salts showed only minor differences from that of the bile salts of the grass carp *Ctenopharyngodon idella* Val. which we have examined and believe to consist almost entirely of 5α-cyprinol sulphate. The sulphate group in this substance is certainly at C-26/27, since a side-chain terminal sulphate group is required to form the anhydro compound, and the i.r. spectrum shows the characteristic bands of the allocholic acid nucleus with unsubstituted –OH groups at C-3α, C-7α and C-12α. Since a sulphate ester grouping at the end of the side chain in 5α-cyprinol sulphate gives an i.r. spectrum very closely similar to that of 5α-chimaerol sulphate, the latter too is probably a C-terminal ester; i.e. the sulphate group is on C-26.

To determine the stereochemistry at C-25 in 5α-chimaerol we reduced samples of the epimeric 3α,7α,12α-trihydroxy-5β,25ξ-cholestan-26-oic acids

(Bridgwater, 1956), given to us by Dr. T. Briggs, with lithium aluminium hydride. The 25*S* (Bridgwater's 25L) acid m.p. 203.5°C, $[\alpha]_D$ +39° gave a non-crystalline 5β,25*S*-cholestane-3α,7α,12α,26-tetrol, $[\alpha]_D$ +25°, and the 25*R* (25D) acid m.p. 183°C, $[\alpha]_D$ +27°, gave the known 5β,25*R*-cholestane-3α,7α,12α,26-tetrol, m.p. 201°C, $[\alpha]_D$ +36°.

EXPERIMENTAL

General. Except where otherwise stated, methods were as described by Bridgwater *et al.* (1962) and Haslewood & Tökés (1969). Elementary microanalyses were by Dr F. B. Strauss, Oxford.

Samples were converted to TMS ethers for g.l.c. with a series 104 (model 24) dual flame ionization chromatograph (W. G. Pye and Co. Ltd., Cambridge, U.K.), with a coiled glass column (length 152cm, internal diameter 3.5mm) packed with deactivated acid-washed Supersorb (100–120mesh; British Drug Houses Ltd., Poole, Dorset, U.K.) coated with a phenylmethyl siloxane polymer (O.V. 17, 3%, Horning *et al.* 1967). TMS ethers (probably involving all hydroxyl groups in the samples treated) were prepared as follows (Horning *et al.* 1967): the sample (50μg) was dissolved with methyl cholate (12.5μg as internal standard) in bistrimethylsilyl acetamide (25μl, Phase Separations Ltd., Queensferry, Flintshire, U.K.), and after the addition of trimethylchlorosilane (5μl) as catalyst, were left about 17h at approx. 22°C. Dilution with methylene chloride (25μl) gave a solution of convenient concentration for direct injection (of 1μl samples) on to the g.l.c. column. This method gave good and reproducible results with purified bile alcohols and crude mixtures from bile salts, unlike other methods for making TMS ethers (including the use of hexamethyldisilazane and trimethylchlorosilane), which often failed with crude mixtures. At 274°C and with a carrier gas (argon) flow of 50ml/min, retention times of TMS ethers relative to methyl TMS-cholate (retention time 20.4min=1) were: 5α-chimaerol, 1.42; 5β-chimaerol, 1.59; 5α-cyprinol, 1.80; 5β-cyprinol, 2.05; anhydro 5α-chimaerol, 1.20; anhydro 5α-cyprinol, 1.75; methyl allocholate, 0.875.

Preparation of bile salts. Bile from *Catostomus commersoni* (6 specimens, average wt. about 250g, from the Mississippi River) was diluted with excess of ethanol. The filtered mixture was evaporated to dryness and the residue washed with light petroleum (b.p. 40–60°C) and triturated with methanol. The mixture was filtered and evaporated to obtain bile salts (0.98g) as a light brown friable solid. On t.l.c. in the system amyl acetate–acetic acid–water (7.5:7.5:3.0, by vol.) this gave a major spot with the mobility of 5α-cyprinol sulphate and a minor spot running at the same rate as taurocholate.

Grass carp (*Ctenopharyngodon idella*; 1 specimen, wt. approx. 5kg, from Malacca, Malaya) similarly gave bile salts (3.7g) which showed a closely similar pattern on t.l.c. The i.r. spectra of *C. commersoni* and *C. idella* bile salts in KBr were identical except for the relative heights of some of the bands in the region 9–11μm.

Dioxan–trichloroacetic acid cleavage. C. commersoni bile salts (200mg) were acetylated and the product was cleaved with dioxan–trichloroacetic acid as previously described (e.g. Haslewood & Tökés, 1969) except that a 1% (w/w) solution of trichloroacetic acid in dioxan was used. After working up, sulphate (79mg of $BaSO_4$) was obtained and the neutral organic product was dissolved in ethanol (5ml) with 5M-NaOH (0.5ml) and heated under reflux for 1h. Evaporation of solvent in nitrogen left a residue which was washed with water and collected. This product was dissolved in methanol and the filtered solution evaporated to give a solid (127mg) that on paper chromatography in system G_3, di-isopropyl ether–heptane–acetic acid–water (6:4:7:3 by vol.), and t.l.c. in the system ethyl acetate–acetic acid–water (17:2:1, by vol.) gave a major spot that ran at almost the same rate as 5α-cyprinol, and two other faint spots. On g.l.c. the TMS ethers gave a major peak with a retention time of 1.42 relative to methyl TMS-cholate and a small shoulder with approximately the retention time of TMS-5α-cyprinol. The cleavage product (127mg) was purified by heating under reflux with acetone and filtering the solution from the little insoluble material that remained. Evaporation left a white residue which with ethyl acetate gave ordered 'globules' that were collected, washed with cold ethyl acetate and dried *in vacuo* over $CaCl_2$. This 5α-*chimaerol* [5α-*cholestane*-3α,7α,-12α,24(+),25S-*pentol*, II] was apparently solvated; it had m.p. 234–235°C, $[\alpha]_D^{24}$ +33.5±1° (*c*1.1 in ethanol) and gave a positive (purple) Hammarsten (HCl) test, a single spot on t.l.c. in the system described above and a single peak on g.l.c. Its i.r. spectrum in KBr closely resembled that of 5α-cyprinol but there was a noticeable difference in the relative dimensions of the bands with maxima at about 9.7 and 9.9μm (Found: C, 70.3; H, 10.3; $C_{27}H_{48}O_5 \cdot \frac{1}{2}H_2O$ requires C, 70.3; H, 10.6%). A solution of this material (50mg) in pyridine (0.5ml) with acetic anhydride (0.5ml) was left for 17h at about 20°C. The product was diluted with water and excess of 2M-HCl and ether-extracted twice. The ether was washed with water, aqueous ammonia and water, dried with Na_2SO_4 and evaporated to a gum (50mg) which crystallized with light petroleum (b.p. 40–60°C). Recrystallization from mixtures of this solvent, benzene and ether and from aqueous ethanol finally gave fine white needles (28mg) of 5α-*chimaerol* 3,7,24,26-*tetra-acetate* m.p. 141–143°C, with an i.r. spectrum very like that of 5α-cyprinol 3,7,26,27-tetra-acetate (Found: C, 67.6; H, 8.7; $C_{35}H_{46}O_9$ requires C, 67.8; H, 9.0%).

Similar treatment of *C. idella* bile salts (50mg) gave a neutral product (26mg) that g.l.c. showed to be mainly 5α-cyprinol with a minor constituent having a retention time (TMS derivative) relative to methyl TMS-cholate of 1.47.

Alkaline hydrolysis. C. commersoni bile salts (206mg) in 0.25M-NaOH (8ml) were heated in a metal bomb for 17h at 125±1°C. The neutral insoluble product was collected and washed with water; the aqueous portion was treated with 2M-HCl and excess of NaCl and, after refrigeration, 'bile acids' were collected and washed with water. These gummy 'bile acids' (0.4mg, approx. 0.2% by wt. of the bile salts) were methylated and the product converted to TMS ethers; g.l.c. gave a sharp peak with the retention time of methyl TMS-allocholate and a smaller broad peak with a retention time (max.) rather longer than that of methyl TMS-cholate. The final acidic aqueous liquors with 0.5M-$BaCl_2$ (10ml) gave $BaSO_4$ (52mg).

On paper chromatography in system G_3 the neutral

organic product (101mg) gave a major spot with the mobility of anhydro 5α-cyprinol and a minor one with that of 5α-cyprinol; the product probably also contained some unhydrolysed sulphate ester, and it was purified by boiling under reflux with ethyl acetate. The mixture was filtered and the solvent evaporated. The residue crystallized slowly from ethyl acetate–acetone to give large colourless prisms (32mg) of *anhydro-5α-chimaerol* (III) m.p. 221–222°C (decomp.), $[\alpha]_D^{24}+31.8\pm2°$ (*c* 0.68 in ethanol), with an i.r. spectrum in KBr very like that of anhydro 5α-cyprinol (Haslewood, 1967). (Found: C, 75.0; H, 10.75; $C_{27}H_{46}O_4$ requires C, 74.6; H, 10.6%). On t.l.c. in the system isopropyl acetate–acetic acid–water (15:3:1, by vol.) this product had a mobility slightly less than that of anhydro 5α-cyprinol. On t.l.c. in the same system a less pure product gave an additional faint spot with a mobility slightly less than that of 5α-cyprinol; after conversion to TMS ethers, g.l.c. of this product gave a definite peak with the retention time of TMS-5α-cyprinol.

Bile salts of *C. idella* (0.5g) dissolved in 0.5M-NaOH (5ml) were heated in a metal bomb at 200±1°C for 17.5h. Neutral material (363mg) and acids (5.4mg) were obtained as described above. G.l.c. showed the acids to consist largely of allocholic acid. Neutral material (240mg) was separated on Celite (30g) in the system benzene–light petroleum (b.p. 80–100°C)–ethanol–water (5:2:5:2 by vol.). Moving phase was collected as 83 fractions (5ml each) and the main peak (90–115ml) contained anhydro 5α-cyprinol (86mg), identified by t.l.c. and g.l.c. Minor peaks (eluted at 75–80ml and 125–130ml) contained material (6.6mg and 3.4mg respectively) with i.r. spectra rather different from that of anhydro 5α-cyprinol and not showing the broad trimethylene oxide band at about 10.4μm. Material (89mg) stripped from the column with methanol (200ml) was shown by g.l.c. (by Mrs. P. A. Howes) to contain 5α-cyprinol with a more polar contaminant. 5α-Chimaerol and its anhydro derivative were not detected.

Oxidation of C. commersoni *bile salts.* These (50mg) were oxidized with 20% CrO_3 (0.2ml) in acetic acid (0.5ml) and the product was isolated and heated with HCl (6ml of approx. 0.25M) as described by Haslewood & Tökés (1969). This treatment gave $BaSO_4$ (20mg) and organic material (3mg) that precipitated as a brown gum completely soluble in cold aqueous ammonia and showing at least 5 spots (visualised by the method of Usui, 1963) on t.l.c. in the system ethyl acetate–acetic acid–water (17:2:1 by vol.), none with the mobility of 3,7,12-trioxo-5α-cholan-24-oic acid.

Preparation of 5β,25ξ-cholestane-3α,7α,12α,26-tetrols. 3α,7α,12α-Trihydroxy-5β,25D(25*R*)-cholestan-26-oic acid (25.7mg) was methylated and lithium aluminium hydride (50mg) was added gradually to the resultant gum in tetrahydrofuran (3ml) at room temperature. The resultant gel was heated on a steam bath for 1h under reflux, cooled, treated with ice and excess of 2M-HCl and extracted with ether–ethyl acetate (1:1, v/v) three times. Evaporation left crystals of 5β-25*R*-cholestane-3α,7α,12α,26-tetrol (23.9mg) m.p. 200–201°C, $[\alpha]_D^{22}+36\pm1°$ (*c* 1.35 in ethanol) (Found: C, 73.1; H, 10.9; $C_{27}H_{48}O_4\cdot\frac{1}{2}H_2O$ requires C, 72.7; H, 11.1%).

Similar treatment of the 25L (25*S*) epimer of the above acid gave the 25*S* tetrol as a gum that failed to crystallize and had $[\alpha]_D^{22}+25\pm2°$ (*c* 1.62 in ethanol).

On t.l.c. in the system isopropyl acetate–acetic acid–water (15:3:1, by vol.) neither tetrol showed more than traces of impurities.

DISCUSSION

Chemical and biochemical. The evidence for formula (II) for 5α-chimaerol is very convincing although conversion to a known steroid has not been achieved. The stereochemistry at C-24 and C-25 can be assessed from M_D values as follows. Bridgwater, Haslewood & Watt (1963) discussed chimaerol (5β-chimaerol) and applied Bridgwater's (1956) value (±34.5°) for the contribution at C-25 in the 3α,7α,12α-trihydroxy-5β,25ξ-cholestan-26-oic acids; they assumed that reduction of –COOH to $-CH_2OH$ would have little effect on M_D. This assumption is unjustified (see the Results section) because the contribution to M_D at C-25 in the chimaerols is more correctly assessed as ±24.5° and in the opposite optical sense; the M_D values for the epimeric 5β,25ξ-cholestane-3α,7α,12α,26-tetrols (V) are +108° and +157°. Masui & Staple (1967) prepared the C-24 epimers of 5β-cholestane-3α,7α,12α,-24-tetrol and found that asymmetry at C-24 contributed ±32.5° to M_D. Cholic acid has M_D +150° and a pure specimen of allocholic acid has M_D +112° (Mitra & Elliott, 1968): thus ΔM_D 5β→5α is −38°. The calculated values of M_D for the C-24/25 epimers of 5α-chimaerol if vicinal effects are ignored are thus +(108−38) or +(157−38) ±32.5°, i.e. +37.5, +86.5, +102.5 and +151.5°. If vicinal effects are sufficiently unimportant our 5α-chimaerol, M_D +153°, is 24(+), 25(+), i.e. 24(+), 25*S*. For 5β-chimaerol, the calculated values of M_D for C-24/25 epimers are +75.5, +124.5, +140.5 and +189.5°. Bridgwater *et al.* (1963) found M_D +187°; hence this substance is also 24(+), 25*S*.

Thus both 5α- and 5β-chimaerol have the same configuration at C-25 as the 3α,7α,12α-trihydroxy-5β,25*R*-cholestan-26-oic acid which is formed in mammalian systems (Berséus, 1965; Mitropoulos & Myant, 1965) from cholesterol originally by stereospecific hydroxylation at C-26. The 25*S* epimer of this acid was isolated from frogs and from certain crocodilians (Shah, Staple & Rabinowitz, 1968); conceivably it was formed by racemization during the alkaline hydrolysis involved in its preparation from bile. No such racemization seems possible when chimaerols are isolated by the dioxan–trichloroacetic acid method.

Biological. Bile salts consisting mainly of the sulphate of 5α-cyprinol and those containing chiefly 5α-chimaerol sulphate can be viewed as resulting from parallel evolution; both types seem equally biogenetically primitive and their chemical similarity suggests that they are functionally equivalent. Taxonomically it would be pleasing if the

Cyprinidae and Catostomidae possessed 5α-cyprinol and 5α-chimaerol respectively, but apparently they do not. When we told Dr. T. Briggs of our results, he kindly informed us that he could identify 5α-chimaerol in the bile of *Catostomus plebeius*, that another sucker, *Carpiodes carpio*, had principally tauroallocholate, and a third, *Ictiobus cyprinellus*, had 5α-cyprinol sulphate as its chief bile salt. We have not found 5α-chimaerol in cyprinid bile, but a wide survey of this and of catostomids will be necessary to assess the taxonomic significance of the distribution of 5α-cyprinol and 5α-chimaerol. As 5α-chimaerol is already oxidized at C-24, biochemically it seems a much better evolutionary precursor of allocholic acid than does 5α-cyprinol. This opinion is supported by Dr Briggs' results on bile acids from *Carpiodes carpio* and by our findings on bile acids from *Catostomus commersoni*.

The occurrence of 5α-chimaerol suggests that C-24 oxidation occurred as an early step in bile salt evolution, perhaps before the division into Chondrichthyes and Osteichthyes; it survives (with a C-5β configuration) in all the chondrichthyeans so far examined but has not been detected in osteichthyeans except in the ostariophysians discussed above.

We thank Dr J. B. Carey for help in the collection of *Catostomus commersoni* bile, and Dr C. F. Hickling, Tropical Fish Culture Research Institute, Malacca, Malaya, for *Ctenopharyngodon idella* bile (for an account of their diet and digestion see Hickling, 1966). We thank Dr T. Briggs, University of Oklahoma Medical Center, for permission to refer to his unpublished work and for a generous gift of C-25 epimeric trihydroxy-5β-cholestan-26-oic acids. We thank Mrs P. A. Howes for some preliminary g.l.c. experiments and the Science Research Council for support.

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APPENDIX

Nuclear-Magnetic-Resonance and Mass-Spectral Examination of the Principal Bile Alcohol from *Catostomus commersoni* and its Anhydro Derivative

By LÁSZLO TÖKÉS
Institute of Organic Chemistry, Syntex Research, Palo Alto, Calif. 94304, *U.S.A.*

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On the basis of i.r. and chromatographic evidence 5α-cholestane-3α,7α,12α,24ξ,26-pentol (I) and 24ξ,26-oxido-5α-cholestane-3α,7α,12α-triol (IV) structures have been proposed for the principal cleavage and alkaline-hydrolysis products of the bile salts from *Catostomus commersoni* (Anderson & Haslewood, 1969). N.m.r.- and mass-spectral analyses of these substances and the tetra- (II) and penta-acetate (III) derivatives of (I) provided supporting evidence for the structural assignments.

EXPERIMENTAL

The n.m.r. spectra were measured by Miss J. Tremble and Dr M. L. Maddox on a Varian HA-100 spectrometer with tetramethylsilane as internal reference. The solvents are shown in Table 1. The mass spectra were recorded by Mr J. W. Smith on an Atlas CH-4 mass spectrometer equipped with an EFO-4B ion source. The ionization potential was maintained at 70 eV.

The preparation of samples (I), (II) and (IV) was reported by Anderson & Haslewood (1969). The penta-acetate derivative (III) was prepared by storing a solution