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Comparative Studies of 'Bile Salts'

18. THE CHEMISTRY OF CYPRINOL*

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The bile alcohol cyprinol has been isolated from four species of fish of the family Cyprinidae and was shown by feeding experiments on captive carp to be very probably made *in vivo* from cholesterol (Haslewood, 1955, 1959). The infrared-absorption spectrum of cyprinol was recognized as showing the presence in the molecule of the nucleus of allocholic acid and chromic oxidation gave a mixture

probably containing 3,7,12-trioxoallocholic acid (Briggs & Haslewood, 1962).

Cyprinol was also made from carp bile by Hoshita (1962), who isolated, after alkaline hydrolysis, the diacetate of anhydrocyprinol. Hoshita (1962) oxidized the acetylated anhydrocyprinol with chromic acid at 70°: the product after hydrolysis was found to contain allocholic acid and also a substance that had the mobility on paper chromatograms of 25-homocholic acid. Lithium

* Part 17: Bridgwater, Haslewood & Watt (1963).

aluminium hydride reduction of anhydrocyprinol gave a product with the mobility on paper of $3\alpha, 7\alpha, 12\alpha, 26$ -tetrahydroxycoprostanane. On the evidence provided by these experiments, Hoshita (1962) suggested that cyprinol had the formula (II). In this Laboratory, we had obtained chimaerol, probably $3\alpha, 7\alpha, 12\alpha, 24\xi, 27$ -pentahydroxy- 25β -coprostanane, from fish bile, and since this substance had almost the same mobility on paper as cyprinol we thought that the latter might be the allo (5α) epimer of chimaerol, a likely possibility on biogenetic grounds (Bridgwater, Haslewood & Watt, 1963).

We now report evidence that establishes that cyprinol has, in fact, structure (II), i.e. $3\alpha, 7\alpha, 12\alpha, 26, 27$ -pentahydroxycholestane.

RESULTS

Cyprinol, m.p. 242° , $[\alpha]_D + 29^\circ$, was obtained from carp bile salts by acid hydrolysis and also after cleavage of the sulphate with dioxan-trichloroacetic acid at room temperature (Bridgwater, Briggs & Haslewood, 1962); it could be purified as the tetra-acetate. Cyprinol tetra-acetate was also isolated from the bream, *Abramis brama*. Cyprinol sulphate was isolated from carp bile salts as a barium salt, analysis of which corresponded to a formula $[C_{27}H_{47}O_4 \cdot O \cdot SO_3^-]_2Ba^{2+}$. Alkaline hydrolysis of carp bile salts gave crude anhydrocyprinol, the crystalline diacetate of which had m.p. 138° [Hoshita (1962) gives m.p. 158°]. The infrared-absorption spectrum of cyprinol (Fig. 1a) shows bands at about $9.7, 9.9$ and 11.2μ , characteristic of the allocholic acid nucleus; the spectrum of (non-crystalline) anhydrocyprinol (Fig. 1b) also shows

these bands, and, in addition, strong absorption at about 10.4μ , characteristic of a trimethylene oxide ring (Cross, 1961). Chromic oxidation of cyprinol gave a mixture that probably contained $3,7,12$ -trioxoallocholic acid (dehydroallocholic acid), but whose chief component proved, after purification, to be $3,7,12$ -trioxoallo- 25 -homocholic acid (III), the methyl ester of which we prepared after an Arndt-Eistert extension of the side chain of $3\alpha, 7\beta, 12\alpha$ -trihydroxyallocholic acid (IV).

Finally, examination of the proton-magnetic-resonance spectrum of cyprinol tetra-acetate by Dr A. D. Cross (Appendix to this paper) gave conclusive evidence that cyprinol is represented by structure (II). The native bile salt is thus the monosulphate ester of compound (II). Since alkaline hydrolysis of cyprinol sulphate gives anhydrocyprinol (V), containing a trimethylene oxide ring, the bile salt must have formula (I). Cyprinol tetra-acetate is the $3,7,26,27$ -tetra-acetate of compound (II) and anhydrocyprinol diacetate the $3,7$ -diacetate of compound (V).

EXPERIMENTAL

General. The methods were those of Bridgwater *et al.* (1963). Paper chromatography (ascending) with acetic acid-water (7:3, v/v) as stationary and 'amyl acetate' (Bridgwater *et al.* 1962)-heptane (1:1, v/v) as moving phase gave the following approximate R_F values: cyprinol, 0.31; anhydrocyprinol, 0.61; cholic acid, 0.47; deoxycholic acid, 0.71; scymnol, 0.20; anhydroscymnol, 0.35.

Cyprinol and its derivatives

Carp bile salts. Gall bladders of 40 freshly killed carp (*Cyprinus carpio*, 'mirror' variety; average wt. about 1 kg.) were cut under a large excess of ethanol-methanol (19:1, v/v). Filtration and evaporation left a residue which was dried *in vacuo* over $CaCl_2$. The solid was broken up and washed with cold light petroleum (b.p. $30-60^\circ$). Re-drying gave the bile salts (7.86 g.) as a dark greenish solid.

Cyprinol sulphate (barium salt). The above bile salts (2.0 g.) in water (5 ml.) were treated with 0.5M- $BaCl_2$ (15 ml.). After refrigeration, the product was collected, washed with water and dried *in vacuo* over $CaCl_2$ and also in an oven at 100° . The final product (1.05 g.) was a tan-coloured powder, m.p. about 170° (decomp. with evolution of gas). A solution made by warming this substance (200 mg.) with aq. 30% (v/v) ethanol (4 ml.) was treated with aq. 10% (w/v) Na_2CO_3 (0.5 ml.) and ethanol (0.2 ml.). The white precipitate was collected, washed with water and dried at 100° and *in vacuo* over $CaCl_2$. The yield of $BaCO_3$ was 32.5 mg. [Found: Ba, 11.3. $(C_{27}H_{47}O_4 \cdot O \cdot SO_3^-)_2Ba^{2+}$ requires Ba, 11.4%].

Acid hydrolysis of bile salts. Carp bile salts (1.0 g.) were dissolved in water (37.5 ml.). After the addition of N-HCl (12.5 ml.) the mixture was heated on the steam bath with occasional swirling. After 21 hr. of heating, the mixture, containing much brown gelatinous material, was poured into water (150 ml.), and extracted with three 50 ml. portions of ethyl acetate-butanol (3:1, v/v). The combined

Fig. 1. Infrared-absorption spectra in potassium bromide: (a) cyprinol; (b) anhydrocyprinol.

extracts were evaporated *in vacuo*, leaving a residue (521 mg.). The water phases were concentrated and treated with excess of 0.5M-BaCl₂. The washed BaSO₄ weighed 234.2 mg.

Cyprinol tetra-acetate. The crude extract (519 mg.) from acid-hydrolysed bile salts (above) was dissolved in pyridine (3.0 ml.), acetic anhydride (2.0 ml.) was added and the mixture was left at room temperature for 23 hr. The solution was poured into water (100 ml.), and the acetylated product extracted three times with ether. The ether was washed with N-HCl, aq. NaHCO₃ and water. The NaHCO₃ and water washes were combined and extracted with ether. The combined ether layers were distilled, and the residue was dried by evaporation with benzene to yield a gum (562 mg.). This crude cyprinol tetra-acetate was dissolved in ether-benzene (1:1, v/v) and placed on a column of neutralized alumina (14 g.) made up in the same mixture. The column was eluted with ether-benzene (1:1, v/v) (25 ml.), ether (25 ml.) and methanol-ether (1:99, v/v) (100 ml.). Fractions (5 ml.) of the last solvent mixture were collected, and those containing the product (35–55 ml.) were pooled and evaporated, leaving a residue (302 mg.) that crystallized from ether. The yield was 156 mg. Recrystallization from ether gave *cyprinol tetra-acetate*, double m.p. 110.5° and 137.5–139°, $[\alpha]_D^{26} + 10 \pm 1^\circ$ (c 1.6 in ethanol) [Found: C, 67.4; H, 8.8. C₃₅H₅₆O₉ requires C, 67.7; H, 9.1%]. The tetra-acetate could be used to prepare a pure sample of cyprinol.

Oxidation of cyprinol tetra-acetate (50 mg.) in acetic acid (1 ml.) with 20% (w/v) CrO₃ (0.1 ml.) for 15 min. at room temperature gave, after dilution and ether extraction in the usual way, a neutral gum (34 mg.) that did not crystallize and had $[\alpha]_D^{23} + 31 \pm 2^\circ$ (c 1.9 in ethanol).

By mild acetylation, followed by purification on alumina columns, cyprinol tetra-acetate was similarly isolated from the products of acid hydrolysis of the bile salts of the tench

(*Tinca tinca*), roach (*Rutilus rutilus*), chub (*Leuciscus cephalus*) and bream (*Abramis brama*), and also from 'wild-type' carp (*Cyprinus carpio*) and 'mirror' carp kept for a year on a diet consisting only of butcher's meat with small supplements of liver. This feeding experiment was done (with the assistance of Mr N. Bedow) in a tank lit artificially and containing no algae or other macroscopic living material.

Cyprinol. The crude extract (521 mg.) from acid-hydrolysed bile salts (above) was separated on Hostalen S [Farbwerke Hoeschst A.-G., Frankfurt (M), Germany] (45 g.) as described by Bridgwater *et al.* (1962). The solvent system was methanol-water-chloroform-2-ethylhexan-1-ol (52:48:5:5, by vol.), i.e. a slightly modified system of Norman (1953). Fractions (5 ml.) of moving (methanol-water) phase were collected; cyprinol (197 mg.) was eluted by 250–340 ml. It was crystallized by warming with acetone, giving *cyprinol* as colourless prisms, m.p. 242–244°, $[\alpha]_D^{23} + 29 \pm 2^\circ$ (c 1.7 in ethanol) (Found: C, 71.7; H, 10.9. C₂₇H₄₈O₅ requires C, 71.6; H, 10.7%).

Cleavage of bile salts with dioxan-trichloroacetic acid. Carp bile salts (1.0 g.) were dissolved in a mixture of acetic acid (0.5 ml.) and acetic anhydride (0.5 ml.). The solid dissolved after heating on a steam bath for about 2 hr. Further acetic anhydride (4.5 ml.) was added, and heating continued another 2 hr. Methanol (5 ml.) was then added and the mixture evaporated on the steam bath under nitrogen; it was finally heated *in vacuo* over NaOH. Methanol was again added, and the evaporation procedure repeated twice. The residue was dissolved in 4.0 ml. of a 40% (w/v) solution of freshly distilled anhydrous trichloroacetic acid in anhydrous dioxan, with warming (at 50–60° for 30 min.) to promote solution. Anhydrous dioxan (12 ml.) was added and the mixture left for 4 days. The mixture was evaporated under nitrogen, with warming (at 60–70°), to small volume. A solution of 4.5 g. of KOH in aq. 80% (v/v)

ethanol (approx. 5 ml.) was added, and the black mixture refluxed for 2 hr. After dilution with water (150 ml.) and acidification with 5*N*-HCl the mixture was extracted with several portions of ethyl acetate-butanol (3:1, v/v). Evaporation gave a dark residue (553 mg.) that was purified by reversed-phase partition-column chromatography, as described above, to yield cyprinol (181 mg.).

Alkaline hydrolysis of bile salts; anhydrocyprinol. Carp bile salts (1.0 g.) were dissolved in 2.5*N*-NaOH (10 ml.), sealed in a bomb and heated for 16 hr. at 112°. The contents of the bomb were rinsed out with water and diluted to 100 ml.; 5*N*-HCl (about 3 ml.) was added to adjust the pH to 10–11. The mixture was extracted with ethyl acetate (four 25 ml. portions), the extracts were washed with water (two 25 ml. portions), the water washes were combined with the original water phase and the whole was extracted with butanol (four 25 ml. portions). The butanol extracts were washed with water (two 25 ml. portions) and the water washes re-extracted once with butanol (15 ml.). The ethyl acetate extracts thus contained hydrolysis products of low polarity; the butanol extracts contained residual hydrolysis products that failed to be extracted by ethyl acetate, together with unhydrolysed bile salts.

Paper chromatography (cf. Sjövall, 1955), with the system 'amyl acetate'-heptane (1:1, v/v) as the moving phase and acetic acid-water (7:3, v/v) as the stationary phase, revealed large quantities of unhydrolysed bile salts in the butanol extract. This was evaporated and the hydrolysis (at 125°) and extraction process were repeated. The butanol extract from the second hydrolysis still contained some unchanged bile salts and small quantities of hydrolysis products. It was not treated further. Ethyl acetate extracts from both hydrolyses were combined and evaporated, and the residue (229 mg.) was shown by paper chromatography to contain two major products, the more polar of which was identified as cyprinol.

The product was separated on a reversed-phase partition column as described above. The effluent peak corresponding to cyprinol contained only 20 mg. The column was therefore washed with methanol, and the residue (207 mg.) after evaporation was passed through another column of Hostalen S (36 g.) with methanol-water (29:21, v/v) as the moving phase, and chloroform-heptane (24 ml.; 9:1, v/v) as the stationary phase (cf. Bergström & Sjövall, 1951). The substance (82 mg.) appearing as a broad peak at 360–600 ml. of effluent was identified (by paper chromatography) as the less-polar product of alkaline hydrolysis (anhydrocyprinol). This substance did not crystallize; its infrared-absorption spectrum is shown in Fig. 1 (b).

Anhydrocyprinol diacetate. Anhydrocyprinol (70 mg.), isolated after partition chromatography as described above, was treated with acetic anhydride and pyridine as described for cyprinol. The crude product was purified by adsorption on alumina and elution with 1% (v/v) methanol in anhydrous ether. The purified product (78.7 mg.) was crystallized twice from light petroleum (b.p. 95–100°), giving *anhydrocyprinol diacetate* (54.7 mg.), m.p. 133–136°, raised by recrystallization to 135–138.5° (Found: C, 72.15; H, 10.05. $C_{21}H_{50}O_6$ requires C, 71.8; H, 9.7%). Hydrolysis gave anhydrocyprinol with the same infrared-absorption spectrum (Fig. 1b) as the compound before acetylation.

Chromic oxidation of cyprinol. Cyprinol (73 mg.) was dissolved in acetic acid (4.0 ml.), and a 20% (w/v) solution

of CrO_3 in aq. 90% (v/v) acetic acid (0.5 ml.) was added dropwise over a period of 15 min. After a further 15 min., more 20% CrO_3 solution (0.2 ml.) was added and the oxidation mixture left for 45 min. Water (0.2 ml.) sufficient to dissolve the precipitate was added, and the oxidation allowed to proceed for another 45 min. The reaction was terminated 2 hr. after the first addition of CrO_3 by the addition of methanol (0.1 ml.). When the solution became green it was poured into water (50 ml.), NaCl was added (precipitate formed) and the mixture extracted with ethyl acetate (10 ml.). NaCl was added as needed to break emulsions. The combined extracts were evaporated and dried by evaporation with ethanol, leaving a pale-green oil (62 mg.). This was treated with 0.5*N*-NaOH (2.5 ml.) and insoluble material was filtered off. The clear solution was diluted with water (to 10 ml.), acidified (white precipitate formed) and extracted five times with ethyl acetate (2 ml. portions). The washed extracts, combined with one re-extraction of the water washings, were evaporated and dried by evaporation with ethanol leaving a residue (51 mg.) containing acidic material.

The acidic material was separated as described for cyprinol on a column of Hostalen S (4.5 g.). The moving phase (44–78 ml.) eluted crystalline material (8.7 mg.), m.p. 191–207°, which was recrystallized from light petroleum-acetone (twice) and ethanol-water to give a product, m.p. 204–206.5°, that on methylation with diazomethane gave crystals identical (mixed m.p.; infrared-absorption spectrum; R_f on paper chromatograms) with methyl aldehyde-25-homocholate, described below.

Preparation of methyl aldehyde-25-homocholate

3 α ,7 β ,12 α -Trihydroxyallo-25-homocholanic acid (Arndt-Eistert reaction). 3 α ,7 β ,12 α -Trihydroxyallocholanolic acid (380 mg.) (Anderson & Haslewood, 1962) was acetylated by the perchloric acid method in the usual way, and the product, recovered after extraction with ether, was dissolved in benzene (12 ml.) and boiled for 3 hr. under reflux with thionyl chloride (2 ml.). The mixture, after standing overnight, was evaporated on the steam bath, and the residue was twice diluted with anhydrous benzene (10 ml.) and re-evaporated, finally under vacuum, to remove excess of thionyl chloride. The gum obtained was dissolved in benzene (4 ml.), and an excess of an ethereal solution of diazomethane (cooled to –5°) was added. The mixture was allowed to warm to room temperature and left for 20 hr. Evaporation gave a gum, which was dissolved in anhydrous 2-methylpropan-2-ol (10 ml.). The addition of 10% (w/v) silver benzoate in triethylamine (0.5 ml.) (Newman & Beal, 1950) at 80° caused an immediate precipitate of silver and evolution of nitrogen. After 4 hr. on the steam bath, the mixture was diluted with methanol (20 ml.) and filtered through neutralized alumina (2.5 g.) to remove the silver. The residue obtained by evaporation was hydrolysed by boiling for 1.5 hr. in ethanol (20 ml.) and 5*N*-NaOH (3 ml.). Dilution with water (20 ml.) and evaporation of the ethanol gave insoluble neutral material (60 mg.), recovered by extraction with ether. Acidification of the aqueous solution gave an acid (approx. 300 mg.) (presumably containing 3 α ,7 β ,12 α -trihydroxyallo-25-homocholanic acid), recovered in the usual way, that failed to crystallize.

Methyl aldehyde-25-homocholate. The above acid (approx. 300 mg.) in acetic acid (8 ml.) was treated with

20% CrO_3 (0.6 ml.) with cooling. The initial mixture was diluted with water (3 vol.). Extraction three times with ethyl acetate yielded a gum (260 mg.) which was esterified with diazomethane, and the product in benzene was separated on neutralized alumina (7 g.). Elution was as follows. Fraction I (eluent: 100 ml. of benzene), 28.7 mg.; fraction II (eluent: 200 ml. of ether), 23.4 mg.; fraction III (eluent: 150 ml. of ethyl acetate), 171.8 mg. The total eluted was 224 mg. Fraction III from ether-light petroleum gave gummy crystals (101 mg.) of m.p. $145\text{--}180^\circ$. With cold ether this material gave small white prisms (55 mg.), m.p. $185\text{--}206^\circ$ (decomp.), which were purified by recrystallization from aq. ethanol and from ether to give a product (30 mg.) of constant m.p. $199\text{--}209^\circ$ (decomp.). This product still showed two spots on paper chromatograms and for final purification was hydrolysed to the acid. This was crystallized once from aq. ethanol, after which re-methylation gave *methyl allodehydro-25-homocholate* (*methyl 3,7,12-trioxalolo-25-homocholanate*) as small white prisms, homogeneous on paper chromatograms, m.p. (depending on the rate of heating) $204\text{--}211^\circ$ (decomp.), not depressed by the compound [m.p. $206\text{--}211^\circ$ (decomp.)] made by methylation of the acidic substance isolated after chromic oxidation of cyprinol, as described above (Found: C, 71.8; H, 9.0. $\text{C}_{28}\text{H}_{38}\text{O}_5$ requires C, 72.5; H, 8.9%).

Paper chromatography of methyl esters of 3,7,12-trioxo acids. Methyl esters of dehydro acids were found to separate satisfactorily in the solvent system di-isopropyl ether-light petroleum (b.p. 80–100°)–acetic acid–water (5:5:7:3, by vol.) used ascending. The spots were developed by dipping in Zimmermann (alkaline 3,5-dinitrobenzene) reagent and drying in a cold air current, and were intensified by redipping the dried paper in fresh reagent and redrying. The following typical R_F values were obtained: methyl aldehydehydrocholate, 0.33; methyl dehydrocholate, 0.38; methyl aldehydehydro-25-homocholate, 0.39; methyl dehydro-25-homocholate, 0.40. The product obtained by the oxidation of cyprinol ran at the same rate as methyl aldehydehydro-25-homocholate.

DISCUSSION

Chemical. Previously reported evidence for the formula of cyprinol rested almost entirely on paper-chromatographic and spectroscopic findings; allocholic acid was the only substance of known constitution identified after degradation experiments (Hoshita, 1962). Our preparation of alodehydro-25-homocholeic acid (III) and its identity with a substance made by oxidation of cyprinol provides formal proof of the carbon skeleton to C-25 and of the position of the nuclear hydroxyl groups. Our assignment of the α -configuration to these groups rests on an infrared-spectroscopic comparison of cyprinol with allocholic acid, and is also supported by the nuclear-magnetic-resonance findings. The above evidence does not provide proof of the positions of the remaining hydroxyl groups, or, indeed, of the nature of the molecule from C-25. Proof of this nature is supplied by the nuclear-magnetic-resonance results, and in summary the evidence that cyprinol is (II) is very strong,

although it ought to be supported by partial synthesis. Oxidation of compound (II) could give rise to compound (III) by the sequence:

The fact that cyprinol and chimaerol (as well as their anhydro compounds) run at almost the same rate on paper chromatograms emphasizes the difficulties to be expected in isolating bile alcohols from many species. Since the 5α - and 5β -isomers are also not clearly separated on paper, mixtures may be encountered which could contain up to four components very difficult to separate that might be expected on biogenetic grounds to be present in the bile. We found that, unlike the parent alcohols, the 5α - and 5β -dehydro (trioxo) acids and their esters were readily separated on paper, as suggested by work with Celite columns (Anderson, Haslewood & Wootton, 1957): these findings agree with those of Barton & Jones (1943) on sterols and ketones derived from them.

We have found it possible to recognize the infra-red-spectral characteristics of the allocholic acid nucleus in the bile alcohols (cyprinol and ranol) in which this occurs, but there is considerably more variation in the spectra than is the case in bile alcohols having the corresponding 5β (cholic acid) nucleus.

Biological. As remarked (Haslewood, 1955), the finding of cyprinol in the bile of Cyprinidae indicates that these fishes are more 'ancient' than has, apparently, been previously supposed. The fact that cyprinol is an allo compound may give further emphasis to this view, and may, in addition, be useful in tracing the origins of this very large freshwater group. It seems unlikely that further evolution of cyprinol could give rise to the 5 α -epimer of chimaerol, for this would involve reduction (at C-26) and reoxidation (at C-24). Thus the chemical nature of their principal bile alcohol clearly separates the Cyprinidae from fishes containing chimaerol or its so far undiscovered 5 α -epimer.

Although we have not examined the other constituents of the bile salts of Cyprinidae, it seems clear that a marked variation exists, some species having less cyprinol and a considerably larger acidic fraction than is found in the carp. It seems possible that more detailed and more extensive examination of the chemistry of cyprinid bile salts will lead to results of great interest to students of fish evolution.

SUMMARY

1. Cyprinol, $C_{27}H_{48}O_5$, m.p. 244° , $[\alpha]_D + 29^\circ$, was isolated by acid hydrolysis or by dioxan-trichloroacetic acid treatment of the bile salts of the carp *Cyprinus carpio*. It was found in carp fed for a

year on meat only, and also in the bile salts of four other fishes (bream, chub, roach, tench) of the family Cyprinidae. Alkaline hydrolysis of carp bile salts gave anhydrocyprinol, $C_{27}H_{46}O_4$, isolated as the diacetate.

2. Chromic oxidation of cyprinol gave a mixture containing 3,7,12-trioxoallo-25-homocholanic acid, the methyl ester of which was also prepared after Arndt-Eistert extension of the side chain of 3 α ,7 β ,12 α -trihydroxyallocholanic acid.

3. From the above evidence and from the results of infrared spectroscopy and especially of nuclear-magnetic-spectral measurements made by Dr A. D. Cross (Appendix to this paper), cyprinol is believed to have structure (II), i.e. 3 α ,7 α ,12 α ,26,27-pentahydroxycholestane. The biological significance of its occurrence in cyprinid bile is briefly considered.

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APPENDIX

A Nuclear-Magnetic-Resonance Spectral Study of Cyprinol

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Cyprinol (Ia) and its tetra-acetate (Ib) have been examined by nuclear-magnetic-resonance spectroscopy.

EXPERIMENTAL

Nuclear-magnetic-resonance spectra were recorded on a Varian A-60 spectrometer with deuteriochloroform and pyridine as solvents and tetramethylsilane as an internal reference. Results are expressed as chemical shifts in τ units (Tiers, 1958), and coupling constants, J , as cycles/sec. Accuracies are of the order of $\pm 0.02\tau$ for the chemical shift and ± 0.5 cycle/sec. for J . Pyridine was purified by storage over potassium hydroxide, followed by filtration and distillation to give a solvent that showed no resonances in the frequency range 0-330 cycles/sec. (4.5-10.0 τ).

RESULTS AND DISCUSSION

Proton resonances that could be assigned with certainty are collected in Table 1. The insolubility of cyprinol precluded examination in deuteriochloroform solvent, and both deuterated and non-

deuterated samples gave barely satisfactory spectra in pyridine solution, in contrast with previous successes with this solvent (Slomp & MacKellar, 1960; Cross, 1960, 1961). The angular methyl-proton resonances only were clearly defined.

Cyprinol tetra-acetate, deuterated at the sole remaining hydroxyl group, furnished a spectrum in which two resonances of equal intensity, totalling 4 protons, were prominent. These resonances were interpreted as being composed of two superimposable doublets at 5.93 τ (J , 5.5 cycles/sec.) each doublet corresponding to the two equivalent protons of a $CH_2 \cdot O \cdot CO \cdot CH_3$ group attached to a non-asymmetric carbon atom bearing the one proton to which they are coupled. One equatorially-oriented proton on a carbon atom bearing a hydroxyl group was observable only as background resonance to the $CH_2 \cdot O \cdot CO \cdot CH_3$ doublets. Resonance multiplets for two separate protons in the environment $CH \cdot O \cdot CO \cdot CH_3$ were coalesced at 4.87-5.20 τ , the half-band width (12 cycles/sec.) being indicative of their equatorial orientation.