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Comparative Studies of Bile Salts

16-DEOXYMYXINOL, A SECOND BILE ALCOHOL FROM HAGFISH

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

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1. Material containing the less polar sulphate previously noticed in hagfish bile salts gave, after dioxan–trichloroacetic acid cleavage, 16-deoxymyxinol [3β,7α,-26(27)-trihydroxy-5α-cholestane]. 2. Anodic coupling of 3β-hydroxy-5β-cholanoic acid and the mixed half esters of DL-methylsuccinic acid, followed by lithium aluminium hydride reduction, yielded 3β,26(27)-dihydroxy-5β-cholestane. 3. 16-Deoxymyxinol, the third known bile alcohol having the 3β-hydroxy-5α-hydrogen configuration, poses again the question of how the 3β-hydroxyl group of cholesterol can be 'retained' in biosynthesis of primitive bile salts.

The principal bile salt in two hagfish species, *Eptatretus stoutii* (= *Bdellostoma*) and *Myxine glutinosa*, is the 3,26(27)-disulphate ester of myxinol, 3β,7α,16α,26(27)-tetrahydroxy-5α-cholestane (Haslewood, 1966; Cross, 1966; Anderson, Haslewood, Cross & Tökés, 1967). A second component of the bile salts was provisionally identified as a sulphate of 16-deoxymyxinol (Anderson *et al.* 1967); we now describe the isolation and chemistry of this deoxymyxinol and also the partial synthesis of 3β,26(27)-dihydroxy-5β-cholestane, a compound we made for comparative purposes when the hagfish bile alcohols were thought to be derivatives of 5β-cholestane.

RESULTS

In both hagfish species mentioned, the residues from which disodium myxinol disulphate had been crystallized showed on paper chromatograms a spot corresponding to a less polar sulphate. Material from *Myxine glutinosa* giving this spot was cleaved by the dioxan–trichloroacetic acid method to yield gums containing myxinol and also less polar alcohols. Separation on Celite gave crystalline 'deoxymyxinol', m.p. 219° and $[\alpha]_D$ +13°, in a yield of about 2% of the bile salts. Deoxymyxinol gave a digitonide; its i.r. spectrum, in potassium bromide, between 6·5 and 13·0 μm. so closely resembled that of 3β,7α-dihydroxy-5α-cholestane (Fig. 1) as to lead us to propose to Dr L. Tökés that it was 3β,7α,26(27)-trihydroxy-5α-cholestane (16-deoxymyxinol, I); Dr L. Tökés's report on this suggestion forms the Appendix to this paper.

We were unable to isolate identifiable material after chromic oxidation of residues containing the (supposed) sulphate of deoxymyxinol; however, the polarity on chromatograms of the second sulphate mentioned above suggests that 16-deoxymyxinol, like myxinol, might occur in the bile salts as a disulphate ester. The polarity of 16-deoxymyxinol on paper chromatograms was much less than that of myxinol or the contaminant mentioned by Haslewood (1966); this unidentified contaminant could of course be an artifact made from myxinol by the dioxan–trichloroacetic acid cleavage procedure.

Anodic coupling of 3β-hydroxy-5β-cholanoic acid and the mixed half esters of DL-methylsuccinic acid, prepared as described by Bridgwater (1956),

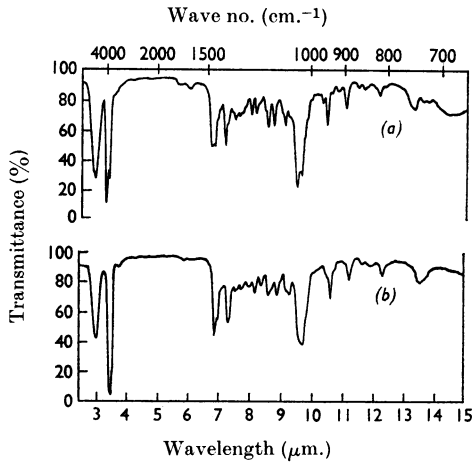
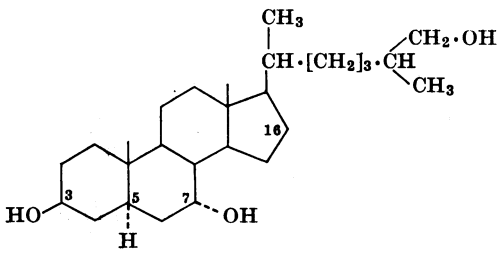
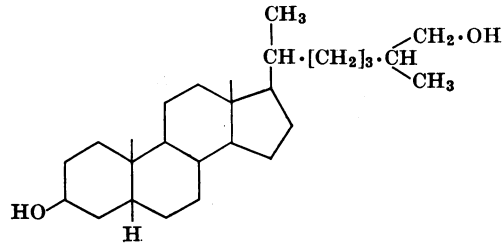


Fig. 1. Infrared spectra, in KBr, of (*a*) 16-deoxymyxinol (I) and (*b*) 3β,7α-dihydroxy-5α-cholestane.



(I)



(II)

gave a neutral product that was reduced by lithium aluminium hydride to yield among other products a crystalline substance having the properties expected of 3β,26(27)-dihydroxy-5β-cholestane (II).

EXPERIMENTAL

General. Methods and analyses were as described by Bridgwater, Briggs & Haslewood (1962). T.l.c. was done on films (thickness, 0·25mm.) of silica gel G (E. Merck A.-G., Darmstadt, Germany).

Isolation of 16-*deoxymyxinol.* The bile salts from 100 gall-bladders of *Myxine glutinosa* gave, on crystallization from ethanol, crude myxinol disulphate (775mg.) and a gum (1·16g.) on evaporation of the mother liquors. The gum was acetylated and treated with dioxan–trichloroacetic acid reagent, and the ether-soluble product was hydrolysed as described by Haslewood (1966). The product, isolated by ethyl acetate extraction, was a yellow gum (130mg.), which on t.l.c. in benzene–propan-2-ol–acetic acid (30:10:1, by vol.) (Eneroth, 1963) appeared to contain two major components in addition to myxinol. An attempt to separate this gum (65mg.) on Celite (10g.) in the system benzene–light petroleum (b.p. 80–100°)–ethanol–water (3:4:5:2, by vol.) was only partly successful. Elution of the principal fractions was as follows (fraction no.; ml. of moving phase; mg. eluted): I, 12–24, 22·5; II, 26–40, 9·2; III, 42–68, 7·6; IV, 70–112, 9·5. Fraction II contained some 16-deoxymyxinol, together with less polar material, and fraction IV contained 16-deoxymyxinol and myxinol. The column was stripped with ethanol (2×50ml.), giving only myxinol (11·9mg.) on evaporation of the eluate. Solid recovered from fraction III was mostly 16-deoxymyxinol and was recrystallized from aqueous ethanol to give needles, m.p. 213–216°. Celite separations with other ratios of solvents were no more successful, nor were reversed-phase columns with system F_1 of Norman & Sjövall (1958). All these methods yielded 16-*deoxymyxinol* (I), giving single spots with R_F much greater than that of myxinol on t.l.c. and paper chromatograms. For example, on paper in the system di-isopropyl ether–heptane–acetic acid–water (5:5:7:3, by vol.) myxinol had R_F about 0·14, 16-deoxymyxinol had R_F about 0·70 and the myxinol contaminant mentioned by Haslewood (1966) had R_F about 0·22. 16-Deoxymyxinol crystallized from aqueous ethanol as solvated needles, m.p. 218–219° and (observation by Dr L. Tökés) $[\alpha]_D$ +13±3° (*c* 0·92 in ethanol). After removal of solvent it gave the i.r. spectrum, in KBr, shown in Fig. 1 (Found: C, 75·0; H, 11·1. $C_{27}H_{48}O_3,\frac{1}{2}H_2O$ requires C, 75·4; H, 11·5%). 16-Deoxymyxinol (2mg.) was dissolved by warming with 0·83ml. of a solution (1%, w/v) of digitonin in ethanol–water (2:1, v/v). Water (0·4ml.) was added and a gelatinous digitonide (4·4mg., after drying) was precipitated; this decomposed at about 205° and had an i.r. spectrum entirely dissimilar to that of 16-deoxymyxinol.

Dr L. Tökés observed that the triacetate, made by acetylating compound (I) (4mg.) with acetic anhydride (1ml.) and pyridine (1ml.) at room temperature for 24hr. and purified by t.l.c. in the system ethyl acetate–hexane (1:1, v/v), was a glass (3mg.), referred to in the Appendix.

Preparation of 3β,26(27)-*dihydroxy-5β-cholestane.* 3β-Hydroxy-5β-cholanoic acid (2·7g., prepared as described by Chang & Blickenstaff, 1958) and the mixed methyl half esters of DL-methylsuccinic acid (7g.) were electrolysed in methanol (35ml.), and the neutral reaction products (3·56g.) were isolated as described by Bridgwater (1956). Chromatography on alumina (107g. of type H; Peter Spence Ltd., Widnes, Lancs.) gave a main fraction (1·13g., eluted by 700ml. of ether) that was hydrolysed by refluxing in ethanol (20ml.) and 5M-NaOH (1ml.) for 1·5hr. Evaporation under N_2 left a brown gum, trituration of which with cold ether (3×20ml.) left a white crystalline residue (415mg.), soluble in ethanol (20ml.); addition of aqueous HCl precipitated crystals, presumably of 3β-hydroxy-5β-cholestan-26(27)-oic acid, which were collected and washed with water. The yield, after drying, was 323mg.

This acid (323mg.) was esterified with diazomethane and the resultant gum refluxed for 3hr. in dry ether (20ml.) with $LiAlH_4$ (50mg. initially, plus 20mg. after 1hr.). Isolation of the reduction product with ether, by the usual method, gave a gum (287mg.), which rapidly crystallized with a mixture of light petroleum (b.p. 40–60°) and ether. Recrystallization from ethyl acetate gave colourless needles (109mg., m.p. 150–152°) of 3β,26(27)-*dihydroxy-5β-cholestane* (II) giving only one spot on t.l.c. and having $[\alpha]_D$ +25·6±2° (*c* 2·07 in ethanol) (Found: C, 79·5; H, 11·7. $C_{27}H_{48}O_2$ requires C, 80·1; H, 12·0%).

Dr A. D. Cross kindly examined the n.m.r. spectrum of this compound and reported as follows.

A 5% (w/v) solution in hexadeuterodimethyl sulphoxide furnished a spectrum in which the resonances for protons on carbon bearing oxygen were ill-resolved. However, a deuterochloroform solution of similar strength was also examined with tetramethylsilane as an internal reference and a Varian HA-100 spectrometer. The spectrum shows 3-proton resonances at δ 0·64 (C-18 H) and δ 0·955 p.p.m. (C-19 H), and a doublet at δ 0·90 p.p.m., *J* 7Hz, which is assigned to a terminal 27-methyl function. Resonance for

a fourth methyl group (at C-21) is not expected to appear as a well-defined doublet and is obscured by other methyl resonances. At lower fields a 2-proton singlet at δ 1·45 p.p.m. is assigned to hydroxylic proton since it disappears from the spectrum on addition of deuterium oxide. An unresolved one-proton multiplet, half-band width 9 Hz, appears at δ 4·08 p.p.m. This resonance is indicative of an equatorial secondary proton on carbon bearing hydroxyl. Only two other protons are clearly differentiated, appearing as two one-proton multiplets at δ 3·43 and δ 3·65 p.p.m. These are assigned to geminal methylene protons of the terminal unit $CHMe \cdot CH_2 \cdot OH$. The higher-field multiplet has a physical appearance of a quartet and the lower-field multiplet that of a triplet. However, further smaller couplings are also evident, indicative of some small longer-range coupling.

DISCUSSION

There seems no reasonable doubt that the deoxymyxinol now isolated has the structure (I). This compound could represent material that had escaped 16-hydroxylation in liver myxinol biosynthesis, or, if, as seems unlikely at present, myxinol is made by 16-hydroxylating intestinal micro-organisms, could be the principle primary hagfish bile alcohol. Biogenetically, the interesting feature of structure (I) seems to us to be the very primitive 3β-hydroxy-5α-hydrogen configuration; 16-deoxymyxinol represents the third known bile alcohol (the others being myxinol and latimerol) showing this structure. If these substances are indeed primary biosynthetic products, a question of interest to biochemists concerned with studies of evolution is whether the 3β-configuration of such bile alcohols is a result of absence or of inactivity of the enzymes responsible for inversion at C-3 in almost all animals making bile salts from cholesterol.

In view of recent demonstrations (Mitroupoulos & Myant, 1967; Wachtel, Emerman & Javitt, 1968) that some C_{24} bile acid biosynthesis can proceed by a route involving side-chain shortening before transformation of the cholesterol ring-structure, it is noteworthy that in 16-deoxymyxinol we have a substance that one might expect if bile salt biosynthesis in hagfish proceeded by the first-elucidated pathway, beginning with 7α-hydroxycholesterol (Danielsson & Tchen, 1968).

To determine whether hagfish bile salts contain any 3α-hydroxy alcohols, and thus show signs of incipient 'modernization', it will be necessary to investigate the still unidentified substances in the bile alcohol fractions.

The authors thank Dr Finn Walvig (University of Oslo Biological Station, Drøbak, Norway) for a generous gift of *Myxine glutinosa* bile, Dr A. D. Cross (Syntex Research, Palo Alto, Calif., U.S.A.) for an n.m.r. examination and report on 3β,26(27)-dihydroxy-5β-cholestane and Dr O. Wintersteiner for a sample of 3β,7α-dihydroxy-5α-cholestane.

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APPENDIX

Nuclear-Magnetic-Resonance and Mass-Spectral Study of 16-Deoxymyxinol

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

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16-Deoxymyxinol and its triacetyl derivative were examined by n.m.r. and mass spectroscopy.

Experimental. The n.m.r. spectra were recorded by Miss J. Tremble on a Varian HA–100 spectrometer with deuterochloroform solutions, unless otherwise indicated, and tetramethylsilane as internal reference. The chemical shifts, δ, are accurate to $\pm$0·01 p.p.m. and the coupling constants, J, to $\pm$0·5 Hz. The mass spectrum was measured by Mr J. W. Smith on an Atlas CH–4