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Comparative Studies of 'Bile Salts'

17. A BILE ALCOHOL FROM *CHIMAERA MONSTROSA**

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The chimaeras (subclass Holocephali) are fishes that may be survivors of a group whose ancestors were early elasmobranchs (Moy-Thomas, 1939; Romer, 1945). The chemical nature of the bile alcohol, scymnol, apparently characteristic of living elasmobranchs has now been almost completely elucidated (Bridgwater, Briggs & Haslewood, 1962*a*), and we wished to find out whether the bile salts of chimaeras would show chemical characteristics compatible with the supposed origin of these fishes.

RESULTS

Alkaline hydrolysis of the bile salts of *Chimaera monstrosa* gave inorganic sulphate and neutral material; no bile acids were identified. The neutral 'bile alcohol' fraction so obtained is described below, and to investigate the 'native' bile alcohols we attempted cleavage of the sulphate esters present by the dioxan–trichloroacetic acid method that had previously yielded scymnol (Bridgwater *et al.* 1962*a*). This process gave, after purification, a crystalline alcohol, 'chimaerol', forming apparently hydrated crystals, m.p. about 180°, $[\alpha]_D$ in ethanol +41·5°. A small fraction with the chromatographic behaviour of scymnol (II) was also obtained.

Chimaerol ran at the same rate on paper chromatograms and showed the same infrared spectrum as the 3α,7α,12α,24ξ,26-pentahydroxy-25ξ-coprostane (I) made by the method of Cross (1961) from anhydroscymnol, and also resembled a substance made by Bridgwater *et al.* (1962*a*) from cholic acid and thought to have formula (I). However, chimaerol is not, apparently, identical with Cross's substance ($[\alpha]_D$ +36°, in pyridine), with which it gave a small melting-point depression and which did not act as a 'seed' for the crystallization of chimaerol. We believe chimaerol to be 3α,7α,12α,-24ξ,26-pentahydroxy-25β-coprostane.

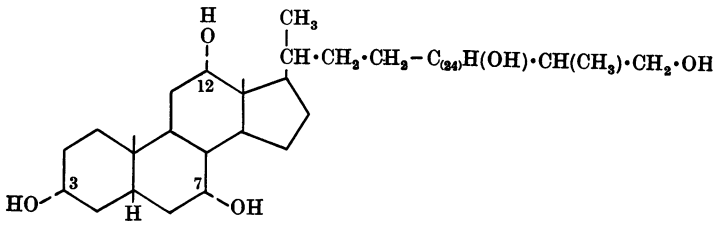
After alkaline hydrolysis at 120°, the main product was a substance with the mobility on paper, in the solvent systems used, of 'anhydrocyprinol', a compound made in this Laboratory by a similar treatment of carp bile salts (see the Discussion section). Purification gave 'anhydrochimaerol' as solvated crystals, m.p. 141°, whose infrared spectrum and other properties suggest that it has structure (III).

EXPERIMENTAL

General

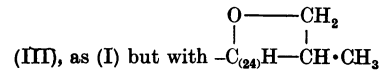
Methods and reagents were as described by Bridgwater *et al.* (1962*a*), and the remarks made by these authors about analytical figures apply. Bile of *C. monstrosa* was collected in ethanol. Evaporation of the filtered ethanol extract from about 100 gall-bladders (some very small) left bile salts (11 g.) as a dark-brown solid. Paper chromatography in 'solvent system T_2' (Bridgwater *et al.* 1962*b*) of this material showed a spot with R_F similar to that of taurocholate, and a second faster-running spot.

* Part 16: Bridgwater, Haslewood & Tammar (1962*b*).



(I)

(II), as (I) but with $-C_{(24)}H(OH)\cdot CH(CH_2\cdot OH)_2$



(III), as (I) but with

(IV), as (I) but with $-C_{(24)}H_2\cdot CH(CH_3)\cdot CO_2H$

Dioxan–trichloroacetic acid cleavage of bile salts

The above bile salts (2·0 g.) with acetic acid (20 ml.) and acetic anhydride (40 ml.) were heated under reflux for 1 hr. after which the condenser was removed and the gently heated solution was evaporated under a nitrogen stream to about 25 ml. After standing overnight at room temperature, the mixture was evaporated to dryness *in vacuo* and dissolved in 24 ml. of anhydrous trichloroacetic acid–dioxan (2:3, w/v). After 30 min., anhydrous dioxan (24 ml.) was added. After 22 hr. the mixture was poured into water (400 ml.) and extracted three times with ethyl acetate. The combined extract (approx. 100 ml.) was washed with water until the washings were neutral, dried (over Na_2SO_4) and evaporated, leaving a resin (0·83 g.) which was dissolved in benzene and separated on a column of alumina (30 g.) made up in benzene. Fractions (100 ml.) of eluate were collected separately, evaporated and examined by paper chromatography. In summary, elution was as follows (fraction no., eluting solvents, total wt. eluted): 1–6, benzene and benzene enriched with ether up to 1:1 (v/v), 100 mg.; 7–10, ether–benzene (1:1, v/v), 100 mg.; 11–21, ether and ether enriched with acetone up to 4:1 (v/v), 300 mg.; 22–29, ether–acetone (1:1, v/v), acetone and acetone–methanol (9:1, v/v), approx. 400 mg. Fractions that were collected when acetone had been standing with alumina for some days were contaminated with products of polymerization, and hence the weights eluted are unreliable in these cases. Resinous material was also not eluted.

Paper chromatography of the above fractions was done with 'solvent system G_5': di-*n*-butyl ether–acetic acid–water (10:7:3, by vol.) In this system, fractions 1–6 showed no spots, and fractions 7–10 and 11–21 gave a single strong spot although some later fractions showed small amounts of substances with different R_F values. Fractions 22–29 consisted of a complex resinous mixture which was further resolved by chromatography on alumina (30 g.). Portions (100 ml.) of acetone–ether (1:1, v/v), acetone, acetone–methanol (9:1, v/v), acetone–methanol (4:1, v/v) and acetone–methanol (1:1, v/v) eluted material (total wt. 130 mg.) that after hydrolysis (as described below) was shown to consist chiefly of the product obtained by hydrolysis from fractions 11–21.

Combined fractions were hydrolysed as follows: the material, dissolved in a minimal volume of ethanol, was heated under reflux for 1–1·5 hr. with 3N-NaOH (10 ml./g.) and, after dilution with water, was extracted with ethyl acetate. Evaporation of the washed and dried extracts left hydrolysis products that were further examined as follows. The hydrolysis product (32 mg.) from fractions 7–10 was separated on paper, as described by Bridgwater *et al.* (1962*b*), in 'solvent system G_5', and thus gave material corresponding to three spots, *A*, *B* and *C*, in order of increasing polarity. The spot *A* material (20 mg.) was crude chimaerol, and the materials (7 and 4 mg.) giving respectively spots *B* and *C* were not identified. The hydrolysis product (118 mg.) from fractions 11–21 was separated on Hostalen and Celite as described below.

Separation on Hostalen. The above hydrolysis product (65 mg.) from fractions 11–21 was separated on a column (10 g.) of 'Hostalen S' (Farbwerke Hoechsst A.-G., Frankfurt, Germany) as described for 'Hostalen W' by Bridgwater *et al.* (1962*a*). The 'solvent system C' of Norman (1953) was used and 2 ml. fractions of eluate were collected automatically, with, in summary, the following results (combined fraction no., amount of eluate, wt. eluted): I, 14–16 ml., 1·0 mg.; II, 32–56 ml., 6·3 mg.; III, 64–98 ml., 37·8 mg. The total weight eluted in main fractions I–III was 44·1 mg. No other single tube contained more than 0·6 mg. and this material was considered to represent 'tailing' and was disregarded. Fraction II showed on paper chromatography a spot corresponding to scymnol and fraction III by paper chromatography and infrared spectroscopy proved to consist largely of chimaerol. However, crystalline material was not obtained in this separation, and this appeared to be due to persistent small amounts of impurities derived from the Hostalen.

Separation on Celite. The hydrolysis product (53 mg.) from fractions 11–21 (above) was separated on Celite

(10 g.) in 'solvent system EC_1' as described by Haslewood (1961): 165 ml. of effluent contained a total of 17·0 mg. of gummy impurities, and a further 40 ml. of effluent gave 2·8 mg. of chimaerol. The column was stripped with acetone (100 ml.) which on evaporation left a solid (32·8 mg.) that, from acetone, gave large colourless crystals (14 mg.), m.p. 178–182°. The mixed m.p. with 26-deoxyscymnol (dihydroanhydroscymnol, m.p. 179–184°) prepared as described by Cross (1961) was 176–182°. Recrystallization from aqueous ethanol gave large crystals of *chimaerol*, m.p. 180–182°, $[\alpha]_D + 41·5 \pm 2°$ (*c* 1·2 in ethanol) (Found: C, 70·7; H, 11·1. $C_{27}H_{48}O_5$ requires C, 71·7; H, 10·6%). This substance had the same infrared spectrum (in KBr) as Cross's (1961) 26-deoxyscymnol; it ran on paper chromatograms at the same rate as this substance and at very nearly the same rate as cyprinol.

Alkaline hydrolysis of bile salts

Chimaera bile salts (0·7 g.) in 3N-NaOH (10 ml.) were heated in a metal bomb at 120° for 16 hr. After dilution, the neutral material was extracted three times with ethyl acetate (approx. 40 ml.) and the washed combined extract was evaporated to give a neutral residue (0·276 g.). On paper chromatography in 'solvent system G_5', this showed a spot with the R_F of chimaerol, another with the mobility of anhydrocyprinol and a third running between chimaerol and anhydroscymnol. For purification, the material was separated on paper in 'solvent system G_5', giving a main product (159 mg.) showing the R_F of anhydrocyprinol and a minor product (36 mg.) not further characterized. The main product (100 mg.) was purified in four lots (25 mg. each) on glass plates (20 cm. × 20 cm.) coated with 'Kieselgel G nach Stahl' (Merck, from Camlab Ltd., Cambridge) in a layer 1·2 mm. thick. The portion of the plate dipped into the solvent [propionic acid–acetic acid (98:2, v/v)] was tapered to prevent crumbling of the layer and running was allowed for about 2 hr. at room temperature. The plate was dried and then covered except for a thin strip, which was sprayed with the phosphomolybdic acid reagent. Heating at 100° showed up the position of organic material (about 10 cm. from the starting line) and a band of silica gel containing this material was scraped off and extracted with methanol. Evaporation left a residue (118 mg.), still containing silica gel. Extraction with acetone removed organic material and the concentrated extract was filtered and evaporated to dryness. The residue in ethyl acetate was filtered, and, after evaporation, the process was repeated in ether. The residue left after evaporation was dissolved in a little benzene, and an excess of light petroleum (b.p. 40–60°) was added. On standing, crystals (m.p. 134–137°) (approx. 12 mg.) appeared, and these were dissolved in acetone. After evaporation, the residue gave, with ether, short colourless needles of *anhydrochimaerol*, m.p. 141–144° (Found: C, 72·95; H, 10·55. $C_{27}H_{46}O_4$ requires C, 74·6; H, 10·6. $C_{27}H_{46}O_4,\frac{1}{2}H_2O$ requires C, 72·9; H, 10·6%). Anhydrochimaerol dissolved readily in acetone or ethyl acetate and it was sparingly soluble in ether, from which it crystallized; it gave no purple colour in the Hammarsten (HCl) test. The infrared spectrum resembled that of chimaerol, but showed much less absorption due to hydroxyl groups, especially at about 9·6 μ. A strong band at about 10·4 μ, not present in the spectrum of chimaerol, may be attributed to the trimethylene oxide ring (Cross, 1961).

DISCUSSION

Chemical. The identification of chimaerol as (I) rests on its elementary analysis and on the identity of its chromatographic behaviour and infrared spectrum with the corresponding properties of the substance of Cross (1961), shown by him to have structure (I). Because of hydration, elementary analysis of substances of this type is frequently unsatisfactory, and although infrared spectra definitely show the nucleus of 3α,7α,12α-trihydroxycoprostane, Bridgwater *et al.* (1962*a*) have pointed out that different substances with this nucleus but also hydroxylated in the side chain may show only small infrared spectral differences. We have found paper chromatography to be a very sensitive method of distinguishing between polyhydroxycoprostanes, and we consider that the evidence for writing chimaerol as (I) is strong, although it falls short of the complete proof that would be given by stereospecific synthesis. We assign to it the 25β-configuration on the following grounds. Bridgwater (1956) prepared 3α,7α,12α-trihydroxy-25α-coprostanic acid with $M_D + 123°$ in ethanol, and the corresponding 25β acid with $M_D + 192°$. Hence the contribution to M_D of C-25 in these acids (IV) is $\pm 34·5°$ and the contribution of the rest of the molecule is $\pm 157·5°$. Our experience suggests that reduction of carboxyl groups to hydroxymethyl groups may not affect M_D much in compounds of this type (e.g. Bridgewater & Haslewood, 1952), and the possible effect of hydroxylation at C-24 may be assessed from the rotation of scymnol (II). Bridgwater *et al.* (1962*a*) found that the 'natural' alcohol had $M_D + 171°$, in ethanol, and partially synthetic scymnol had $M_D + 161°$; the difference is within errors caused by variations in experimental procedure and possible impurities. Taking M_D for scymnol as $+166°$, the difference from the above value ($+157·5°$) for the molecule with C-24 as a methylene group is $+8·5°$, which could perhaps be attributed to the effect of the hydroxyl group at C-24. There is, of course, no proof that the hydroxyl group at C-24 has the same configuration in scymnol and chimaerol, or, indeed, that either substance, as isolated, is optically pure. However, the above considerations do suggest (*a*) that the effect on M_D of hydroxylation at C-24 is small, and (*b*) that chimaerol ($M_D + 187°$) consists mainly of material with the 25β-configuration.

We believe, from unpublished evidence obtained in this Laboratory with Dr T. Briggs, that the bile alcohol cyprinol (Haslewood, 1955) from Cyprinidae is probably the allo (5α) isomer of chimaerol, and that 'anhydrocyprinol', prepared by alkaline hydrolysis of carp bile salts, is the allo isomer of (III). The polyhydroxy allo compounds run at almost the same rate on paper chromatograms as

the corresponding 5β substances (Anderson & Haslewood, 1962), and we therefore think that the rate of movement on paper of anhydrochimaerol supports the opinion that it is (III). This opinion receives further strong support from the infrared spectrum of anhydrochimaerol (see the Experimental section).

Biological. The structure (I) is exactly what would be expected for a biochemical precursor of scymnol, for *C*-terminal hydroxylation would lead directly to this compound. Hence the principal finding of this work is that the chemistry of *C. monstrosa* bile salts does support the view that the living chimaeras are survivors of a group ancestral to those elasmobranchs that contain scymnol. Okuda, Enomoto, Morimoto & Kazuno (1962) have isolated from the bile of the sting ray, *Dasyatis* (*Dasybatis*) *akajei*, a (non-crystalline) substance which is an artifact of the alkaline hydrolysis used and to which they assign the structure (III). This may (depending on configuration at C-24 and C-25) be anhydrochimaerol, made from chimaerol sulphate in the bile salts by the alkaline hydrolysis. Thus *D. akajei* may also contain chimaerol sulphate, and if this is so it emphasizes even more strongly the close relationship between the chimaeras and the other elasmobranch fishes. Anhydroscymnol and cholic acid have also been isolated from the bile of *D. akajei* (Ashikari, 1939).

SUMMARY

1. Bile salts of *Chimaera monstrosa*, when treated with the dioxan–trichloroacetic acid reagent used by Bridgwater *et al.* (1962*a*) for the isolation of scymnol from its sulphate, yielded a new bile alcohol, chimaerol, which is believed to be 3α,7α,12α,24ξ,26-pentahydroxy-25β-coprostane (II). Paper chromatography suggested that scymnol was also present.

2. Alkaline hydrolysis of the bile salts gave anhydrochimaerol (III), an artifact.

3. The structure of chimaerol supports the view that the chimaeras belong to a group which included the ancestors of those elasmobranch fishes that contain scymnol.

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The Metabolism of Glycerol 1-Phosphate in Resistant and Susceptible Houseflies (*Musca domestica* L.) and the Effect of Dieldrin

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Winteringham (1960) found that the amount of glycerol 1-phosphate in the thoracic tissues of adult houseflies was higher under conditions of cyclopropane anaesthesia than under conditions of normal activity. It was suggested that this difference was associated with the change from activity to rest. Previous poisoning with dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-endo-5,8-dimethanonaphthalene) or DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] abolished or reduced this effect of cyclopropane anaesthesia (Winteringham, Hellyer & McKay, 1960).

Anoxia also increases the concentration of glycerol 1-phosphate in many insects, e.g. in *Periplaneta americana* thoracic muscle (Kubista, 1957),