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BILE ACIDS

LIX. PURIFICATION OF 5α-ANHYDROCYPRINOL BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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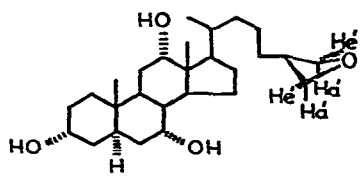
SUMMARY

26,27-Oxido-5α-cholestane-3α,7α,12α-triol can be obtained in a homogeneous state in gram quantities by passing it through one PrepPak-500/Silica cartridge mounted in a Waters Assoc. preparative liquid chromatograph. The elution solvent was methanol–chloroform (1:14). The isolated material was analyzed for purity by several chromatographic means and by elemental analysis, and was finally characterized by the usual spectroscopic means. Gas–liquid chromatography of its trimethylsilyl ether indicated the formation of a tetrakis-trimethylsilyl-26-chloro derivative, in addition to the expected tris-trimethylsilylated substance. The structure of the former compound is deduced from the fragmentation and isotope abundance in its mass spectrum and from chemical principles.

INTRODUCTION

Anhydrocyprinol, 26,27-oxido-5α-cholestane-3α,7α,12α-triol (I), may be obtained from carp bile by alkaline hydrolysis, but it has not been reported as a crystalline substance[1,2]. It is a useful starting material for the preparation of a number of 5α-sterols and bile acids; *e.g.*, 5α-cholest-11-ene-3α,7α,12α-triol, a competitive inhibitor with high affinity for the hepatic microsomal, 12α-steroid hydrox-



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ylase from rabbit[3]; 5α-cholestane-3α,7α,12α-triol, the product obtained during assay of hepatic 12α-hydroxylase with the substrate 5α-cholestane-3α,7α-diol[3–5]; 5α-cholestane-3α,7α,12α,26-tetrol[1,2,6]; 3α,7α,12α-trihydroxy-5α-cholestanoic acid[2,7]; 3α,7α-dihydroxy-5α-cholestanoic acid[2]; and allochenodeoxycholic acid[1,8,9]. This paper gives an account of an efficient method for purification and characterization of 5α-anhydrocyprinol.

EXPERIMENTAL

A Waters Assoc. (Milford, Mass., U.S.A.) preparative liquid chromatograph, Model Prep LC/System 500, containing one PrepPak-500/Silica cartridge (column 1 ft. × 2 in I.D.) and equipped with a Model 504 differential refractometer as the detector was used. The flow-rate was 0.25 l/min.

The following spectroscopic and chromatographic methods have been described[10,11]: melting point, proton magnetic resonance (PMR) and infrared (IR) spectra, thin-layer chromatography (TLC) on plates coated with silica gel H (0.25 mm), analytical high-performance liquid chromatography (HPLC) on a Waters Assoc. Model ALC-201 instrument, fitted with a Model U6K injector and a μBondapak/C_{18} column (30 cm × 4 mm I.D.), eluted with 2-propanol–10 m*M* potassium phosphate, pH 7.0 (230:270) at a flow-rate of 1 ml/min, and with a Model 401 differential refractometer as detector. All solvents and chemicals were Fisher (Pittsburgh, Pa., U.S.A.) reagent grade except 2-propanol, which was Fisher HPLC grade. Elemental analyses were performed by Galbraith Labs. (Knoxville, Tenn., U.S.A.).

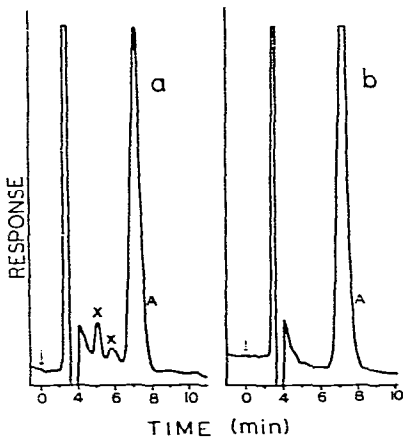
Gas–liquid chromatography (GLC) was carried out isothermally with a F and M Model 402 gas chromatograph fitted with a flame detector and U-shaped silanized glass columns (6 ft. × 4 mm I.D.) packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The following conditions were used: column temperature, 250°; flash heater temperature, 275°; detector temperature, 260°: helium (40 p.s.i.) at a flow-rate of 60 ml/min. A trimethylsilyl (TMS) ether of the appropriate material was prepared as previously reported[12]; the retention time was related to the bis-TMS ether of methyl deoxycholate as 1.00. The absolute retention time of this standard on 3% OV-17 was 17.5 min. Gas chromatography–mass spectrometry (GC–MS) was carried out with an LKB Model 9000 spectrometer, fitted with a silanized coiled glass column (6 ft. × 0.25 in. O.D.) packed with 1% OV-17 on 100–120 mesh Gas-Chrom Q at 250° as reported[12].

Bile was released from the gall bladders of carp indigenous to Missouri streams as obtained from a local wholesale fishery. Ethanol was added to precipitate protein and other insoluble foreign matter. The ethanol extracted was dried and the product was hydrolyzed with 2.5 *N* KOH in an autoclave for 12 h[2]. The hydrolysate was poured into a large volume of water, extracted with ethyl acetate, and the ethyl acetate was removed with a roto-evaporator. The dry crude product was purified by acetic acid partition chromatography[13]. From fractions 40–2 to 40–4[2,13], the desired product was obtained, and purified from acetone or methanol–water in semi-crystalline form[1,2]. The first number in the designation of the fraction refers to the concentration (%) of benzene in a hexane–benzene mixture (equilibrated with the stationary phase, 70% acetic acid) and the second number refers to the fraction in the

respective series, *e.g.* fraction 40–2 is the second fraction eluted with 40% benzene in hexane.

RESULTS AND DISCUSSION

Preparations of 5α-anhydrocyprinol (TLC: $R_F = 0.38$; methanol–chloroform (1:3)) obtained from acetic acid partition chromatography[2,13], are sometimes accompanied by a substantially more polar material ($R_F = 0.05$) and other minor impurities, as demonstrated by the HPLC chromatogram (Fig. 1a). The brown solids obtained by crystallization from methanol–water are incompletely soluble in chloroform. Purified 5α-anhydrocyprinol is obtained on a preparative scale from such preparations by chromatography with Prep LC/System 500 and a mixture of methanol–chloroform (1:14) (Fig. 2) or acetone–benzene (1:1). The solvent compositions selected provided a mobility of the sterol on TLC with R_F between 0.1 and 0.15. The latter solvent mixture was not as satisfactory as the former because of poorer capacity in solubilizing the sample. However, its use was prompted by considerations that the oxetane ring in 5α-anhydrocyprinol might be opened and that the metal components of the instrument might become corroded during prolonged contact with methanol–chloroform, owing to generation of acidic substances, as reported by Ku and Freeman[14] and Gerbacia[15] for solvent mixtures consisting of carbon tetrachloride and alcohol or ether. With methanol–chloroform as eluent, a maximum load of *ca.* 7 g of anhydrocyprinol, obtained by acetic acid parition chro-



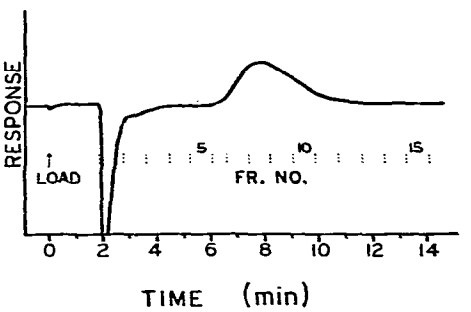


Fig. 1. Purity of 5α-anhydrocyprinol, analyzed by analytical HPLC. A reversed-phase system (μBondapak C_{18}) was used as described in Experimental. The detector response was set at 8 ×. (a) Chromatogram of a sample prior to purification by preparative HPLC; the arrow indicates the point of injection; after solvent peaks, impurities labeled as X appeared, followed by 5α-anhydrocyprinol (A). (b) Chromatogram of 1 mg of purified anhydrocyprinol (A).

Fig. 2. Purification of 1.9 g of 5α-anhydrocyprinol by preparative HPLC. Eluent, methanol–chloroform (1:14); the refractometer setting, 5 ×. Other conditions are as described in Experimental. Fractions of *ca.* 200 ml were collected, as indicated by dashed vertical lines. Purified anhydrocyprinol was obtained in fractions 6–12. The more polar contaminants were eluted in later fractions.

matography, yielded over 50% of purified material and *ca.* 25% of the more polar contaminants. Some mixed fractions containing anhydrocyprinol could be combined and rechromatographed. The crude organic extract from alkaline hydrolysis of carp bile yielded only contaminated anhydrocyprinol if chromatographed directly on Prep LC.

Purified anhydrocyprinol is slightly tan-colored and substantially more soluble in chloroform than the precursor material prior to Prep LC. It is homogeneous in TLC with methanol–chloroform (1:3) and with acetone–benzene (4:1) ($R_F = 0.41$)[2], and in analytical HPLC (Fig. 1b); with a ratio of 300:200 for the solvent mixture, the relative retention volume was 0.81, as related to deoxycholic acid with a retention volume of 6.3 ml. GLC of the TMS derivative of this sterol provides a more complex chromatogram (Fig. 3) as explained below.

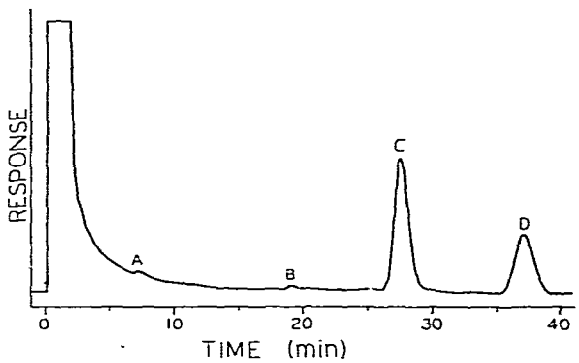


Fig. 3. Gas–liquid chromatogram of the trimethylsilyl derivative of purified 5α-anhydrocyprinol on 3% OV-17. Other conditions are as described in Experimental. Arrow indicates the point of injection. Peak A which follows the solvent peak, was due to an impurity in the trimethylsilylating agent (pyridine–hexamethyldisilazane–trimethylsilyl chloride). Peak B was due to the impurity with $t_{R,rel}$ 1.15. Peak C consisted of anhydrocyprinol tris-TMS ether ($t_{R,rel}$ 1.65). Peak D contained material with the assigned structure III ($t_{R,rel}$ 2.32).

Purified anhydrocyprinol is crystallizable from either methanol–water or ethyl acetate–hexane, the former being preferred because dissolution can be achieved at room temperature. In either case, the sterol precipitates as a gel with extensive trapping of solvent molecules which can be removed almost completely by prolonged drying over phosphorous pentoxide at 100° and 0.01 mm. Elemental analysis of such a sample yields the values C 73.27% and H 10.50%, in agreement with the formula $C_{27}H_{48}O_4 \cdot 0.5\ H_2O$ (C 73.10% and H 10.65%). Various preparations vary widely in their melting points which nonetheless were sharp, *e.g.*, 212–213°, 218–220°, and 228–229°. Such variations are especially conspicuous with crystallized material. Efforts to remove the remaining traces of color by chromatography with grade V alumina (Woelm, Eschwege, G.F.R.) were unsuccessful.

The IR spectrum of purified anhydrocyprinol is essentially the same as reported[1,2]. PMR data obtained in a solution of deuterochloroform containing a trace of deuterated methanol are as follows: $\delta = 0.68$(s) (18-H), 0.76(s) (19-H of 5α-saturated steroids[16,17]), 3.83(m) (7-H)[17], 3.96(m) (12-H)[17], 4.01(m) (3-H)[17], 4.38 (t;$J = 6$ Hz; 2H) ($H^{c'}$) and 4.80 ppm (quartet; $J = 6$ Hz and 8 Hz; 2H) ($H^{a'}$). The

assignment of $H^{a'}$ and $H^{e'}$ in structure I is rationalized as follows: the pseudo-axial protons, $H^{a'}$, which are chemically identical, are more hindered sterically and closer to the lone pair of electrons of the oxygen atom than the pseudo-equatorial protons, $H^{e'}$, and therefore are less shielded. From stick and ball models and based on a modified Karplus curve[18], the dihedral angle $H^{e'}$–C–$H^{a'}$ of *ca.* 125° gives a J value of 6 Hz between $H^{e'}$ and $H^{a'}$. Since $H^{e'}$–C–H_{25} is also 125°, a triplet is expected for $H^{e'}$ atoms with $J \sim 6$ Hz. On the other hand, a dihedral angle of 15° for $H^{a'}$–C–H_{25} suggests a coupling constant of 8 Hz. Thus a quartet with J values of 6 Hz and 8 Hz should be observed for $H^{a'}$ atoms.

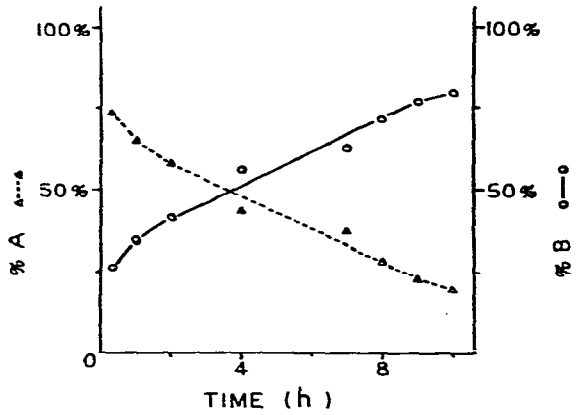
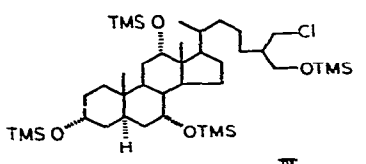
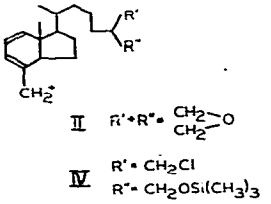


Fig. 4. Extent of formation of compound III (denoted as B in the graph) with time of trimethylsilylation; a simultaneous decrease in the amount of 5α-anhydrocyprinol tris-TMS ether (A) is also shown. Conditions of analysis by GLC are as described in Experimental.

The TMS derivative of 5α-anhydrocyprinol yielded three peaks in GLC (Fig. 3) of $t_{R,rel}$ 1.15, 1.65 and 2.32 relative to the TMS ether of methyl deoxycholate. The peak with $t_{R,rel}$ 1.15 amounts to *ca.* 1% of total material. The peak height of the major component, $t_{R,rel}$ 1.65, decreased with time of reaction while the component with $t_{R,rel}$ 2.32 increased (Fig. 4); a precursor–product relationship is obvious. The substance with $t_{R,rel}$ 1.65 evidently is the tris-TMS ether of anhydrocyprinol, as interpreted from the mass spectral fragmentation. The molecular ion, m/z 650 (0.9%), suffers loss of one to three trimethylsilanol groups (90 a.m.u.), among which the ion m/z 470 [$M - (2 \times 90)$] is the base peak. Each of the above four ions gives rise to fragments due to simple or combined losses of a methyl group, side chain, and part of ring D, as has been observed with the TMS derivatives of methyl esters of allo bile acids[19]. A significant peak is also present at m/z 273 (39.5%) owing to



III



II R'+R" = $CH_2{<}^{CH_2}_{CH_2}{>}O$

IV R' = CH_2Cl
R" = $CH_2OSi(CH_3)_3$

cleavage of the molecular ion across ring *B* (C-9 — C-10 and C-6 — C-7) with loss of the 12α-TMS group as trimethylsilanol (II)[19]. Loss of CH_2O from *m/z* 470 could explain the ion *m/z* 440 (8.6%).

The component with $t_{R,rel}$ 2.32 contained a small ion at *m/z* 758, which could be the molecular ion for the tetrakis-TMS derivative of (25*R*,*S*)-26-chloro-5α-cholestane-3α,7α,12α,27-tetrol (III). Up to four trimethylsilanol groups are eliminated from the ion *m/z* 758 with the ion at *m/z* 578 [$M - (2 \times 90)$] being the base peak. Each of the above ions can undergo loss of a methyl group and the complete side chain. In addition, the ion *m/z* 381 (8.1%) (IV) can be formed from the molecular ion by the loss of 377 a.m.u.[19]. The ions *m/z* 578 and 488 derived from the molecular ion with respective losses of two or three trimethylsilanol groups, give rise to ions at *m/z* 470 and 380, respectively, which can be due to the removal of the 26-Cl and 27-TMS groups with subsequent ring closure to form an oxetane ring. A rough calculation of isotope compositions of the assigned structure III[20], whose formation from an unstable oxetane ring in the presence of both basic (pyridine) and acidic (trimethylsilyl chloride) agents is not unexpected, shows that they are similar to those observed as listed in Table I.

TABLE I

ABUNDANCES OF SEVERAL INTENSE FRAGMENT IONS OF COMPOUND III

Isotope abundance is expressed as percent of the fragment ion. Calculation of isotope abundance is based on the equations[20]:

$$\frac{[A+1]^+}{A^+} = (1.1\% \times \text{No. of C atoms}) + (5.07\% \times \text{No. of Si atoms})$$

$$\frac{[A+2]^+}{A^+} = \frac{1}{200}[(1.1 \times \text{No. of C atoms})^2\% + (5.07 \times \text{No. of Si atoms})^2\%] + (0.2\% \times \text{No. of O atoms}) + (3.31\% \times \text{No. of Si atoms}) + (32.7\% \times \text{No. of Cl atoms})$$

m/z	*Composition*	*A + 1*		*A + 2*	
		Calc.	*Obs.*	*Calc.*	*Obs.*
578	$C_{33}H_{59}O_2Si_2Cl$	46%	42%	47%	39%
381	$C_{22}H_{38}OSiCl$	30%	34%	40%	38%
343	$C_{22}H_{35}OSi$	29%	37%	7%	12%

The GC–MS of the TMS derivative ($t_{R',rel}$ 1.77) of the major component of the more polar contaminants of anhydrocyprinol indicated that it is a pentahydroxylated 5α-cholestane compound with three of the hydroxyl groups located in the nucleus. Although 5α-cyprinol (5α-cholestane-3α,7α,12α,26,27-pentol) is one of the major constituents of bile alcohols from carp[1], an unequivocal identification of the contaminant awaits purification and comparison with a purified sample of 5α-cyprinol.

The reported method of purification of 5α-anhydrocyprinol should simplify the procurement of gram amounts of sterols derived from anhydrocyprinol for biochemical studies.

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REFERENCES

1 T. Hoshita, *J. Biochem. (Japan)*, 52 (1962) 125.
2 S. Y. Kamat and W. H. Elliott, *Steroids*, 20 (1972) 279.
3 R. Shaw and W. H. Elliott, *J. Biol. Chem.*, 254 (1979) 7177.
4 M. M. Mui and W. H. Elliott, *Biochemistry*, 14 (1975) 2712.
5 S. S. Ali and W. H. Elliott, *Biochim. Biophys. Acta*, 409 (1975) 249.
6 T. Hoshita, T. Sasaki and T. Kazuno, *Steroids*, 5 (1965) 241.
7 K. Okuda, M. G. Horning and E. C. Horning, *Proc. 7th Int. Cong. Biochem., Tokyo, 1967*, Vol. IV, Abstracts 721, Sci. Council, Japan, Tokyo.
8 R. J. Blaskiewicz, G. J. O'Neil, Jr. and W. H. Elliott, *Proc. Soc. Exp. Biol. Med.*, 146 (1974) 92.
9 W. H. Elliott, in S. Matern, J. Hackenschmidt, P. Back and W. Gerok (Editors), *Advances in Bile Acid Research*, Schattauer, Stuttgart, 1975, p. 31.
10 R. Shaw and W. H. Elliott, *J. Lipid Res.*, 19 (1978) 783.
11 R. Shaw, J. A. Smith and W. H. Elliott, *Anal. Biochem.*, 86 (1978) 450.
12 W. H. Elliott, L. B. Walsh, M. M. Mui, M. A. Thorne and C. M. Siegfried, *J. Chromatogr.*, 44 (1969) 452.
13 J. T. Matschiner, T. A. Mahowald, W. H. Elliott, E. A. Doisy, Jr., S. L. Hsia and E. A. Doisy, *J. Biol. Chem.*, 225 (1957) 771.
14 A. Y. Ku and D. H. Freeman, *Anal. Chem.*, 49 (1977) 1637.
15 W. E. F. Gerbacia, *J. Chromatogr.*, 166 (1978) 261.
16 R. F. Zürcher, *Helv. Chim. Acta*, 46 (1963) 2054.
17 Y. Shalon, R. Shaw and W. H. Elliott, in preparation.
18 N. S. Bhacca and D. H. Williams, *Applications of NMR Spectroscopy in Organic Chemistry; Illustrations from the Steroid Field*, Holden-Day, San Francisco, 1966, p. 50.
19 W. H. Elliott, in G. Waller (Editor), *Biochemical Applications of Mass Spectrometry*, Wiley, New York, 1972, p. 291.
20 F. W. McLafferty, *Interpretation of Mass Spectra*, Benjamin, New York, 1966, p. 210.