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MICROBIAL DEGRADATION OF DEOXYCHOLIC ACID BY *PSEUDOMONAS sp.* NCIB 10590. CHARACTERISATION OF PRODUCTS AND A POSTULATED PATHWAY

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Abstract—Minor products in the microbial degradation of deoxycholic acid are characterised and a metabolic pathway proposed. In a previous publication[1] we reported the transformation of deoxycholic acid **1** by *Pseudomonas sp.* NCIB 10590, in a mineral salts medium, to a mixture of neutral and acidic steroids. The major neutral product, 12β-hydroxyandrosta - 1,4 - dien - 3,17 - dione **2** and the major acidic product 12α - hydroxypregna - 1,4 - dien - 3 - one - 20 - carboxylic acid **9** were identified. We now report the structures of the minor products and propose a metabolic pathway.

It has been suggested that the microbial transformations of bile acids may be implicated in the aetiology of breast[2] and colon[3] cancer and that these transformations may be particularly important in anaerobic conditions, where nitrate may act as a terminal electron acceptor. Accordingly, the transformations were carried out under aerobic and anoxic conditions, with and without nitrate present, in order to test the possibility that the nature of the terminal electron acceptor may alter the nature and relative proportions of products, in particular those compounds, e.g. phenols, which might prove to be carcinogenic.

RESULTS AND DISCUSSION

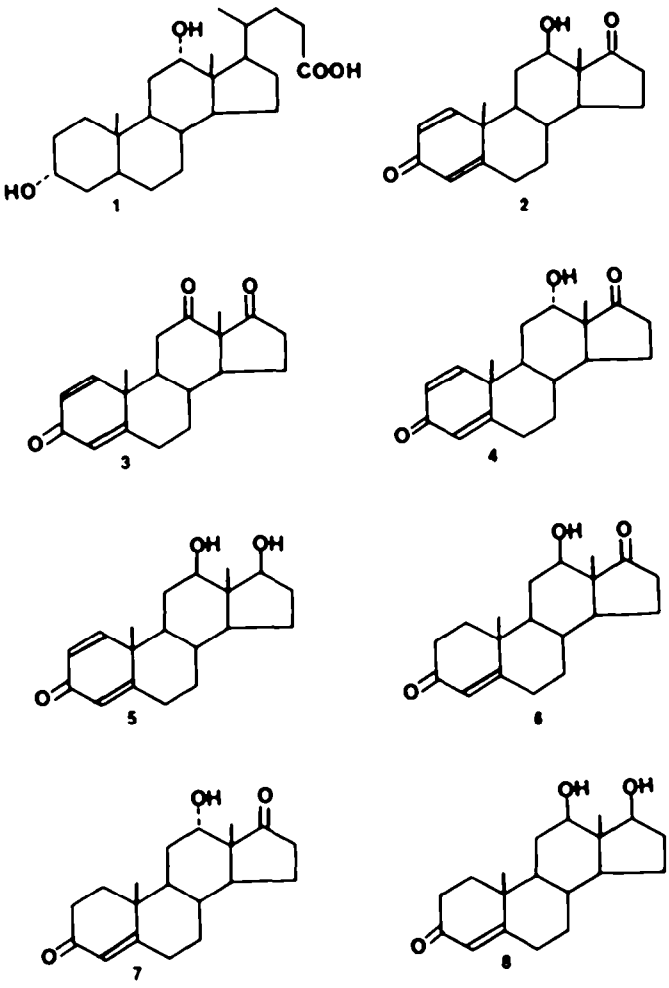
Pseudomonas sp. NCIB 10590 was cultured in a mineral salts medium (100 l.) using sodium deoxycholate as a sole carbon source. After 16 hr the fermentation was terminated by acidification and the products were separated into a neutral and an acidic fraction. The metabolites differed in the nature of the substituent at C-12, the degree of unsaturation of the A ring, and, in the case of the acids, the length of the side chain.

The major neutral compound (17.1% yield) was the previously characterised[1] 12β - hydroxyandrosta - 1,4 - dien - 3,17 - dione **2**. In addition, two other compounds, **4** and **5**, having the 1,4 - dien - 3 - one A ring were isolated. The nature of the A ring was deduced in each case from IR, UV, PMR and mass spectra. Compounds **2**, **4** and **5** were each readily oxidised to the known[1] trione **3**, indicating the same oxygen substitution pattern at C-3, C-12 and C-17. Compound **5** was acetylated easily but was not reduced by sodium borohydride and was therefore classified as a 12,17-diol. This conclusion was supported by IR and mass spectral evidence. The stereochemistry of the hydroxyl groups was deduced from the PMR spectrum: the chemical shift values and splitting patterns at 3.82δ and 3.45δ accorded with the published[4] values for a 17α proton and a 12α proton in 17β-hydroxysteroids and 12β-hydroxysteroids respectively. In the case of the 12α proton the dihedral (Karplus[5]) angles with the 11β and 11α protons are about 180° and 60° respectively, giving rise to a four line multiplet (J = 10 Hz and 5 Hz). The 17α proton bisects the dihedral angle between the adjacent 16α and 16β protons; the resulting pattern is a triplet (J = 8 Hz). Compound **5** was therefore assigned the structure 12β,17β - dihydroxyandrosta - 1,4 - dien - 3 - one.

Compound **4** was isomeric with compound **2**; spectral evidence indicated the same A ring structure and the same D ring ketone (peak at 1740 cm^{-1} in the IR spectrum). Acetylation proceeded readily with acetic anhydride/pyridine and reduction with sodium borohydride gave a diol isomeric with the diol **5** but more polar on tlc. It was concluded therefore, that the only difference between **4** and **2** was in the stereochemistry of the hydroxyl group at C^{-12}. The PMR spectrum contained a triplet (J = 3 Hz) at 4.14δ the position and pattern predicted[4] for a 12β proton in a 12α-hydroxy steroid. The mass spectrum of compound **4** showed an intense ion (the base peak) at *m/e* 82 (*M*-18). In contrast, the 12β-hydroxy isomer **2** gave rise to a rather low intensity ion (relative abundance 15%) at *m/e* 282 in accordance with Spiteller's[6] observation that in the mass spectra of steroids, loss of the elements of water occurs more readily with axial hydroxyl groups than with equatorial hydroxyl groups. Compound **4** was therefore a diastereoisomer of compound **2** with the structure 12α - hydroxyandrosta - 1,4 - dien - 3,17 - dione.

The remaining neutral steroids (**6**, **7** and **8**) showed the same relationship to each other on TLC as did **2**, **4** and **5**. The mass spectrum of each compound showed an intense ion at *m/e* 124 suggesting a steroidal 4-en-3-one.[7] Confirmation of this A ring structure was provided by the immediate development of an intense yellow colouration with isonicotinic acid hydrazide[6] spray, by UV spectra, and in the case of compound **6** by IR and PMR spectra. Oxidation of **6**, **7** and **8** gave the same trione, slightly less polar (on tlc) than androsta - 1,4 - dien - 3,12,17 - trione **3**, suggesting that the substitution pattern was the same in all three compounds. Compound **8** was identical with the sodium borohydride reduction product of **6** but not with that of **7**. All three compounds were easily acetylated, **6** and **7** to monoacetates and **8** to a diacetate. The PMR spectrum of **6**, like that of **2**, included a four line pattern centred at 3.70δ, indicating a C-12 hydroxyl group in the β configuration. This assignment was supported by the presence of a low intensity M-18 ion in the mass spectrum of **6**. In contrast, compound **8** gave rise to low intensity ions at M-18 and M-36. Compounds **6**, **7** and **8** were therefore assigned the structures 12β - hydroxyandrost - 4 - en - 3,17 - dione, 12α - hydroxyandrost - 4 - en - 3,17 - dione and 12β,17β - dihydroxyandrost - 4 - en - 3 - one.

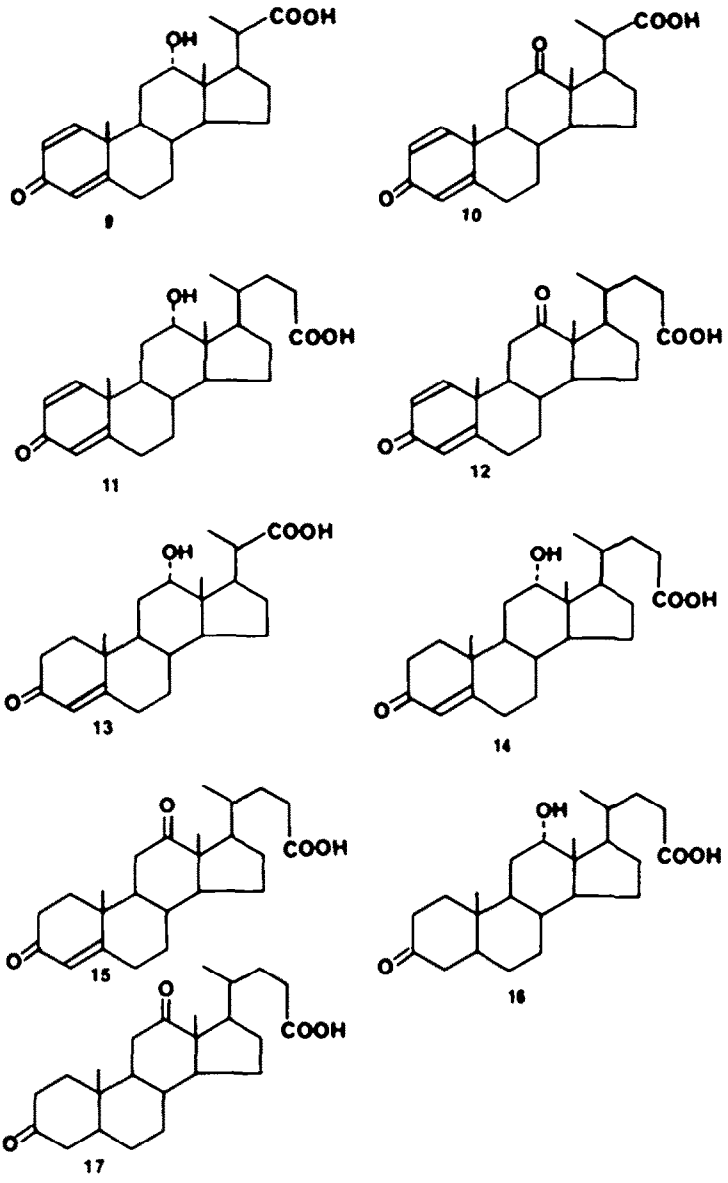
The major acidic metabolite (8.6% yield) was the previously characterised[1] 12α - hydroxypregna - 1,4 - dien - 3 - one - 20 - carboxylic acid, compound **9**. Chromatography of the acid fraction yielded two more acids **11** and **12**, shown by their spectra to have the 1,4 - dien - 3 -



one A ring structure, three acids **13**, **14** and **15** shown by their spectra to have the 4 - en - 3 - one A ring structure and two saturated keto acids **16** and **17**.

The chemical and spectroscopic properties of **11** were almost identical with those of the acid **9**. It was apparent from the UV, IR, PMR and mass spectra that the compounds had identical A ring structures. Treatment of **11** with acetic anhydride/pyridine gave a mono-acetate; oxidation of **11** with Jones' reagent[9] gave a ketone identical with **12**. The IR (peak at 1704 cm^{-1}) and PMR (2 proton multiplet at 2.70–2.50δ) spectra of **12** indicated that the ketone group was at C-12. The PMR spectrum of **11** (triplet at 3.98δ) indicated[4] a 12α-hydroxy group, a conclusion supported by the mass spectrum (intense M-18 peak). The mass spectrum of **11** also enabled the length of the side chain to be determined; this mass spectrum and that of compound **9** both contained an intense peak at *m/e* 267, corresponding to the loss of the elements of water from the molecular ion and loss of the side chain by cleavage of the 17–20 bond.[10] In the case of **11**, the loss of 115 + 18 mass units indicated that the compound had an unchanged bile acid side chain. Its structure was therefore 12α-hydroxy - 3 - oxochola - 1,4 - dien - 24 - oic acid and that of compound **12** was 3,12 - dioxochola - 1,4 - dien - 24 - oic acid. The methyl esters of **13**, **14** and **15** gave UV, IR, PMR and mass spectra in accordance with the 4-en-3-one A ring structure. Compounds **13** and **14** were easily acetylated and oxidised (to a ketone identical with **15** in the case of **14**). The presence of a secondary hydroxyl group was confirmed in each case by IR spectra; position and configuration were established from PMR and mass spectra. The PMR spectra of **13** and **14** each showed a triplet at 4.05δ indicative[4] of a 12α hydroxyl group. This conclusion was supported by the presence of an intense ion at M-18 in the mass spectrum of each compound (as methyl esters). Intense ions at *m/e* 269 (**13**, M-18-87 and **14**, M-18-115) and *m/e* 285 (**15**, M-115) corresponded to the loss of the side chain usually observed[10] in the mass spectra of α,β-unsaturated ketones of cholanic and bisnorcholanic acids. Thus **14** and **15** had the normal bile acid side chain and **13** had the same bis-nor side chain as **9** and its oxidation product **10**.

Compound **13** was therefore 12α - hydroxypregn - 4 - en - 3 - one - 20 - carboxylic acid and compound **14** was 12α - hydroxychol - 4 - en - 3 - one - 24 - oic acid. Compound **15** was 3,12 - dioxochol - 4 - en - 24 - oic acid. The methyl esters of **16** and **17** were shown, using Jones' reagent, to be oxidation products of the starting material. The methyl ester of **16** was obtained by oxidation of methyl deoxycholate under very mild conditions; more vigorous oxidation gave the methyl ester of **17**. Physical constants and spectroscopic data confirmed that **16** and **17** were, respectively, the known compounds 12α -



hydroxy - 3 - oxo - 5β - cholan - 24 - oic acid[11] and 3,12 - dioxo - 5β - cholan - 24 - oic acid.[12]

The crude mixture of metabolites also contained several compounds classified as phenols from their UV spectra and chromatographic behaviour. Further work is necessary to characterise these compounds. It had been established previously[13] that, under anaerobic conditions, oxidation of deoxycholic acid by either *Pseudomonas* NCIB 10590 or *Clostridium paraputrificum* is coupled to reduction of nitrate. In order to investigate this phenomenon under aerobic conditions the experiments described in this paper were repeated using ammonium chloride instead of potassium nitrate as a nitrogen source in the microbial growth medium. The pattern of metabolites produced was not significantly altered. However, under anoxic conditions, significant differences were observed in the pattern of metabolites formed: when potassium nitrate was used as a nitrogen source the pattern of steroidal metabolites was virtually unchanged, with **2** and **9** the major products as before, but no phenolic products were detected. The nitrate ions were reduced to nitrite in the course of the fermentation. When ammonium chloride was used as a nitrogen source, no steroidal products were formed and only phenolic metabolites were detected. It seems likely, therefore, that under anoxic conditions different pathways were used in the presence and in the absence of nitrate ions.

For the aerobic transformations described in this paper it is possible to postulate a degradative pathway by considering the nature of the products formed and by comparison with the known pathways of microbial degradation of other steroids.

The first step is probably oxidation of the 3α-hydroxyl group; no products containing this group have been detected and Hayakawa[14] notes that all microbial metabolites of bile acids having a double bond at C1–C2 or C4–C5, also have a ketone group at C-3. Since no products having a double bond only at C1–C2 have been isolated it seems likely that dehydrogenation at C1–C2 occurs after dehydrogenation at C4–C5.

Hayakawa[14] postulates that for side chain cleavage to take place the bile acid must first be transformed to a 4-en-3-one; the results quoted above support this postulate. In the case of 12α-hydroxysteroids there appears to be a further restriction on side chain cleavage: no products of side chain cleavage between C-17 and C-20 retaining a 12α-hydroxyl group have been isolated. It is probable that for bile acids having a 12α-hydroxyl group, enzymatic cleavage is sterically hindered and that the hindrance is removed when the 12α-hydroxyl group is oxidised or epimerised. The epimerisation at C-12 is not without precedent; a 12α-hydroxysteroid oxidoreductase has been isolated by Skalhegg[15] from *Pseudomonas testosteroni*.

Under aerobic conditions, degradation of the steroid nucleus and formation of phenolic products probably occurs by the pathway described by Sih.[16] The key step in this pathway is 9α-hydroxylation, an oxygen requiring step. It is noteworthy that phenolic products are not formed when the degradation is carried out anaerobically using nitrate as an electron acceptor; the formation of phenolic products when ammonium chloride is used as a nitrogen source is unexplained.

It has been suggested by Hill *et al.*[2] that microbial transformation of bile acids may be important in the aetiology of breast[2] and colon[3] cancer. In order to investigate this possibility the metabolites described in this paper are being subjected to preliminary screening by the Ames[17] procedure.

EXPERIMENTAL

UV spectra were recorded in MeOH on a Pye-Unicam SP1800 recording spectrophotometer and IR spectra in KBr discs on a Pye-Unicam SP1200 spectrophotometer. The PMR spectra were obtained in $CDCl_3$ on a Varian HA-100 at 100 MHz. Microanalyses were done by Butterworth Microanalytical Consultancy Ltd., Teddington. Separation by glc was achieved at 260° using 3% OV-17 on 80/100 mesh "Supelcoport" in a 1.5 m × 3 mm silanised glass column. Retention times were measured relative to 5α-cholestane at a flow-rate of 30 cm^3 min^{-1} nitrogen in a Hewlett-Packard HP5470 instrument. Silyl ethers were prepared by dissolving the previously dried sample in bis-trimethylsilylacetanide (BSA) and heating at 60° for 20 min. All acidic compounds were methylated before injecting into the glc. Purification was achieved by column chromatography on Kieselgel 60,70–230 mesh ASTM (E. Merck, Darmstadt), fractions were eluted with increasing concentrations of CH_3OH in CH_2Cl_2. Tlc analysis and separation was performed on 0.2 mm layers of Kieselgel GF_{254} DC-Fertigplatten (E. Merck, Darmstadt) in the solvent system $CH_3OH:CH_2Cl_2$, 1:9. UV-absorbing components were detected by observation under light of 254 or 340 nm wavelength and all components were finally visualised by spraying the plate with anisaldehyde reagent and heating at 110° for 10 min.[18] Isonicotinic acid hydrazide spray reagent[8] was used to distinguish between 4-en-3-oxo (immediate intense yellow colour) and 1,4-dien-3-oxo-steroids (colourless, becoming yellow).

12-Oxosteroids were prepared by treating an acetone solution of the hydroxysteroid with Jones' chromic acid reagent[9] at 4°. Acidic steroids were methylated with BF_3/methanol. Mps were determined on a Koffler hot stage microscope and are uncorrected.

Degradation under aerobic conditions using potassium nitrate as a nitrogen source

The cells obtained by centrifugation of a ten litre culture of *Pseudomonas* NCIB 10590 were used to inoculate 100 l of medium of composition sodium deoxycholate (100 g), K_2HPO_4 (160 g), KH_2PO_4 (40 g), KNO_3 (100 g), $FeSO_4 7H_2O$ (0.25 g), $ZnSO_4 7H_2O$ (0.25 g), $MnSO_4 4H_2O$ (0.25 g) $MgSO_4 7H_2O$ (10 g), distilled water to 100 l. (final pH 7.2). The culture was incubated at 28° with an aeration of 2 l./min^{-1} and agitation 400 rev min^{-1}. The fermentation was terminated after 16 h by acidification to pH 4 with HCl. Extraction with dichloromethane (4 × 5 l) gave, after drying over $MgSO_4$ and evaporation, 43.4 of tarry residue. This residue was dissolved in warm CH_2Cl_2 (500 cm^3); on cooling, unchanged 1 (10.5 g) crystallised out.

Acidic compounds were extracted from the CH_2Cl_2 solution into 6% $NaHCO_3$ (5 × 300 cm^3). Acidification of the aqueous extract to pH 4 with HCl, followed by extraction with EtOAc, yielded 16.0 g of acidic residue. Half of this residue was methylated, using BF_3/CH_3OH, and the other half was dissolved in hot ethyl acetate. On cooling, the later solution yielded large prisms of 9 (5 g). The methylated mixture gave crystals of the methyl ester of 9 (3.4 g) from CH_3/CH_2Cl_2 and the residue left after crystallisation was separated by repeated preparative tlc in trimethylpentane:acetic acid: ethyl acetate, 45:10:45, to yield the following acids as their methyl esters: 11 (9 mg), 12 (3 mg), 13 (10 mg), 14 (7 mg), 15 (2 mg), 16 (12 mg), 17 (2 mg).

The neutral fraction (16.9 g) on standing in CH_2Cl_2 yielded large prisms of 2 (7.6 g). The remaining mixture of neutral steroids was repeatedly chromatographed on a column of silica gel to give 4 (30 mg) and 5 (150 mg). Further separation, using preparative tlc ($CH_3OH:CH_2Cl_2$) gave 6 (3 mg), 7 (1 mg) and 8 (1 mg), and 2 mg of a tar, λ_{max} 218, 275 nm (226, 295 nm in aqueous NaOH).

Degradation under aerobic conditions using ammonium chloride as a nitrogen source

The experiment was repeated on a one litre scale; all the constituents were in the same relative proportions except potassium nitrate, which was replaced by ammonium chloride (1 g). The fermentation was terminated and extracted as before to give an acidic fraction, 154 mg and a neutral fraction 91 mg. Spectral and chromatographic analysis revealed no phenolic metabolites or products different from those described above. The major products were again compounds 2 and 9.

Degradation under anoxic conditions

Two fermentations, one litre each, were carried out under strict anoxic conditions. The relative proportions of sodium deoxycholate and mineral salts were as described above; in one case KNO_3 was used as a nitrogen source, and in the other ammonium chloride was used. The fermentations were terminated after 14 days. Extraction gave residues of 530 mg and 692 mg respectively. Where nitrogen was present as NH_4Cl, unchanged starting material (500 mg) was recovered together with a mixture of very small amounts of phenolic compounds.

Where KNO_3 was used as a nitrogen source the residue, after separation into neutral (126 mg) and acidic (404 mg) fractions and purification by preparative TLC, gave crystals of 9 (8 mg) and 2 (5 mg). Chromatographic analysis showed the same pattern of products as that produced in the 100 l. aerobic fermentation. Colorimetric analysis, using the method described by Gowan[19] showed that all the nitrate present at the start of the fermentation had been reduced to nitrite.

12β,17β-Dihydroxyandrosta-1,4-dien-3-one (5)

Needles from MeOH/CH_2Cl_2, m.p. 166–167°. (Found: C, 75.22; H, 8.78. $C_{19}H_{26}O_3$ requires 75.50; H, 8.61%); λ_{max} 245 nm (log ε 4.17); IR (KBr disc) 1600, 1616, 1660, 3340, 3480 cm^{-1}; NMR ($CDCl_3$) δ 0.86, 1.22 (6H, two s, C-18 and C-19 protons), 3.45 (1H, 4 line m, J = 5, 10 Hz, C-12 proton), 3.82 (1H, t, J = 8 Hz, C-17 proton), 6.03 (1H, s, slight splitting, C-4 proton), 6.19 (1H, d, showing further splitting, J = 10 Hz, C-2 proton), 7.00 (1H, d, J = 10 Hz, C-1 proton); mass spectrum, *m/e* 302 (12.5); 122 (100), 284 (10), 266 (8); glc R_f 3.4 (OV-17).

12β-Hydroxyandrosta-1,4-dien-3,17-dione (4)

Prisms from MeOH/CH_2Cl_2, m.p. 222–223°. (Found: C, 75.56; H, 8.16. $C_{19}H_{24}O_3$ requires: C, 76.00; H, 8.00%); λ_{max} 244 nm (log ε 4.17); IR (KBr disc) 1600, 1618, 1660, 1740, 3400 cm^{-1}; NMR ($CDCl_3$) δ 0.94, 1.23 (6H, two s, C-18 and C-19 protons), 2.38–2.45 (2H, m, C_{16} protons), 4.14 (1H, t, J = 3 Hz, C-12 proton), 4.65 (1H, s, OH), 6.08 (1H, s, slight splitting, C-4 proton), 6.20 (1H, d, showing further splitting, J = 10 Hz, C-2 proton), 6.96 (1H, d,

j = 10 Hz, C_1 proton); mass spectrum *m/e* 300 (22.5), 282 (100), 121 (95); glc R_f 2.7 (OV-17).

12β-Hydroxyandrost-4-en-3,17-dione (6)

Needles from MeOH (CH_2Cl_2, m.p. 211–213°. (Found. C, 75.82; H, 8.80. $C_{19}H_{26}O_3$ requires: C, 75.50; H, 8.61%); λ_{max} 241 nm (log ε 4.19); IR (KBr disc) 1610, 1655, 1722, 3450 cm^{-1}; NMR ($CDCl_3$) δ 0.98, 1.22 (6H, two s, C-18 and C-19 protons), 2.30–2.50 (2H, m, C-16 protons), 3.70 (1H, 4 line m, J = 5 Hz, 10 Hz, C-12 proton), 5.77 (1H, s, C_4 proton); mass spectrum *m/e* 302 (50), 284 (17.5), 124 (100); glc R_f 2.2 (OV-17).

12α-Hydroxyandrost-4-en-3,17-dione (7)

λ_{max} 242 nm; mass spectrum *m/e* 302 (20), 284 (90), 124 (100); glc R_f 2.3 (OV-17).

12β,17β-Dihydroxyandrost-4-en-3-one (8)

λ_{max} 243 nm; mass spectrum *m/e* 304 (11), 2.86 (6), 268 (5), 124 (100); glc R_f 2.9 (OV-17).

3,12-Dioxopregna-1,4-dien-20-carboxylic acid (10) (*methyl ester*)

Chromic acid (0.65 cm^3, Jones' reagent) was added dropwise to a solution containing 500 mg of 9 in acetone (5 cm^3, distilled from $KMnO_4$) and methylene chloride (cm^3) at 4°. The mixture was held at 4° for 16 h after which time oxidation was shown to be complete by tlc. Excess reagent was decomposed with propan-2-ol (1 cm^3) and the mixture poured into ice–water. The product was isolated in methylene chloride and recrystallised to constant m.p. to give prisms (420 mg) m.p. 200–202°. (Found: C, 74.67; H, 8.01. $C_{23}H_{30}O_4$ requires: C, 74.59; H, 8.11%); λ_{max} 244 nm (log ε 4.18); IR (KBr disc) 1603, 1623, 1657, 1692, 1727 cm^{-1}; mass spectrum *m/e* 370 (13), 283 (15), 121 (100); glc (Me ester) R_f 5.7 (OV-17).

12α-Hydroxychola-1,4-dien-3-one-24-oic acid (11)

Prisms from MeOH/CH_2Cl_2, m.p. (Me ester) 242–244°. (Found: C, 74.76; H, 9.18. $C_{25}H_{36}O_4$ requires: C, 75.00; H, 9.00); λ_{max} (methanol) (Me ester) 244 nm (log ε 4.17); IR (KBr disc Me ester) 3450, 1734, 1660, 1618, 1602 cm^{-1}; NMR ($CDCl_3$ Me ester) δ 0.78, 1.04 (6H, two s, C-18 and C-19 protons), 1.28 (3H, d, J = 6 Hz, 21-CH_3) 3.70 (3H, s, C-24-OCH_3), 4.00 (1H, t, J = 3 Hz, C-12 proton), 6.02 (1H, slight splitting, s, C-4 proton), 6.15 (1H, d, further splitting, J-10 Hz, C-2 proton), 6.96 (1H, s, j = 10 Hz, C-1 proton); mass spectrum *m/e* 400 (2.5), 382 (27.5), 267 (55), 121 (100); glc R_f 12.06 (OV-17).

3,12-Dioxochola-1,4-dien-24-oic acid (12)

Needles from MeOH/CH_2Cl_2, m.p. (Me ester) 208–210°; λ_{max} (methanol) (Me ester) 244 nm (log ε 4.15); IR (KBr disc Me ester) 1730, 1704, 1660, 1622, 1600 cm^{-1}; mass spectrum (Me ester) *m/e* 398 (12), 283 (14), 121 (100).

12α-Hydroxypregn-4-en-3-one-20-carboxylic (13) (*methyl ester*)

Needles from MeOH/CH_2Cl_2, m.p. 231–233°; λ_{max} (methanol) 241 nm (log ε 4.19); IR (KBr disc) 3450, 1702, 1655, 1613 cm^{-1}; NMR ($CDCl_3$) (Me ester) δ 0.80, 1.22 (6H, two s, C-18 and C-19 protons), 1.24 (3H, d, J = 6 Hz, 21-CH_3), 3.64 (3H, s, 22-OCH_3), 4.00 (1H, t, j = 3 Hz, C-12 proton), 5.82 (1H, s, C-14 proton); mass spectrum (Me ester) *m/e* 374 (2), 356 (38), 269 (63), 124 (100).

12α-Hydroxychol-4-en-3-one-24-oic acid (14) (*methyl ester*)

Needles from MeOH/CH_2Cl_2, m.p. 234–235°; λ_{max} (methanol) 241 (log ε 4.18); IR (KBr disc) 3460, 1735, 1660, 1618 cm^{-1}; NMR ($CDCl_3$) δ 0.79, 1.05 (6H, two s, C-18 and C-19 protons), 1.30 (3H, d, J = 6 Hz, 21-CH_3), 3.72 (3H, s, 22-OCH_3); 4.11 (1H, t, J = 3 Hz, C-12 proton), 5.77 (1H, s, C-4 proton); mass spectrum *m/e* 402 (4), 384 (39), 269 (72), 124 (100)

3,12-Dioxochol-4-en-24-oic acid (15) (*methyl ester*)

Needles from MeOH/CH_2Cl_2, m.p. 196–198°; λ_{max} (methanol) 241 nm (log ε 4.17); IR (KBr disc) 1736, 1706, 1665, 1624 cm^{-1}; mass spectrum *m/e* 400 (9), 285 (16), 124 (100).

REFERENCES

[1] P. J. Barnes, J. D. Baty, R. F. Bilton and A. N. Mason, *Tetrahedron* **32**, 89 (1976).
[2] M. J. Hill, P. Goddard and R. E. O. Williams, *Lancet* **ii** 472 (1971).
[3] M. J. Hill, *Am. J. Clin. Nutr.* **27**, 1475 (1974).
[4] J. E. Bridgeman, P. C. Cherry, A. S. Clegg, J. M. Evans, E. R. H. Jones, A. Kasal, G. D. Meakins, Y. Morisawa, E. E. Richards and P. D. Woodgate, *J. Chem. Soc. (C)* 250 (1970).
[5] M. Karplus, *J. Chem. Phys.* **30**, 11 (1959).
[6] E. Zietz and G. Spiteller, *Tetrahedron* **30**, 585 (1974).
[7] H. Budziekiewicz, *Biochemical Applications of Mass Spectrometry*, (Edited by G. R. Waller), Chap. 10, p. 267. Wiley-Interscience, New York (1972).
[8] J. Vaedtke and A. Gajewska, *J. Chromatog.* **9**, 345 (1962).
[9] A. Bowers, T. G. Halsall, E. R. H. Jones and A. J. Lemin, *J. Chem. Soc.* 2555 (1953).
[10] Z. V. Zaretskii, *Mass Spectrometry of Steroids*, Chap. 5, p. 129. Wiley, New York (1976).
[11] A. S. Jones, M. Webb and F. Smith, *J. Chem. Soc.* 2164 (1949).
[12] J. G. Hamilton and J. W. Dieckert, *Arch. Biochem. Biophys.* **82**, 203 (1959).
[13] P. J. Barnes, R. F. Bilton, F. Fernandez, M. J. Hill and A. N. Mason, *Biochem. Soc. Transact.* **3**, 299 (1975).
[14] S. Hayakawa, *Adv. Lipid Res.* **11**, 143 (1973).
[15] B. A. Skalhegg, *Scand. J. Gastroenterology* **9**, 555 (1974).
[16] C. J. Sih and H. W. Whitlock, *Ann. Rev. Biochem.* **37**, 661 (1968).
[17] B. N. Ames, J. McCann and E. Yamasaki, *Mutation Research* **31**, 347 (1975).
[18] D. Kritchevsky, D. S. Martak and G. H. Rotblat, *Anal. Biochem.* **5**, 388 (1963).
[19] S. T. Gowan and K. J. Steel, *Manual for the Identification of Medical Bacteria*, Cambridge University Press (1978).