The degradation of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590 under anaerobic conditions

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The bacterial degradation of cholic acid under anaerobic conditions by *Pseudomonas* sp. N.C.I.B. 10590 was studied. The major unsaturated neutral compound was identified as 12 β -hydroxyandrosta-4,6-diene-3,17-dione, and the major unsaturated acidic metabolite was identified as 12 α -hydroxy-3-oxochola-4,6-dien-24-oic acid. Eight minor unsaturated metabolites were isolated and evidence is given for the following structures: 12 α -hydroxyandrosta-4,6-diene-3,17-dione, 12 β ,17 β -dihydroxyandrosta-4,6-dien-3-one, 12 β -hydroxyandrosta-1,4,6-triene-3,17-dione, 12 β ,17 β -dihydroxyandrosta-1,4,6-trien-3-one, 12 α -hydroxyandrosta-1,4-diene-3,17-dione, 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, 3,12-dioxochola-4,6-dien-24-oic acid and 12 α -hydroxy-3-oxopregna-4,6-diene-20-carboxylic acid. In addition, a major saturated neutral compound was isolated and identified as 3 β ,12 β -dihydroxy-5 β -androstan-17-one, and the only saturated acidic metabolite was 7 α ,12 α -dihydroxy-3-oxo-5 β -cholan-24-oic acid. Nine minor saturated neutral compounds were also isolated, and evidence is presented for the following structures: 12 β -hydroxy-5 β -androstan-3,17-dione, 12 α -hydroxy-5 β -androstan-3,17-dione, 3 β ,12 α -dihydroxy-5 β -androstan-17-one, 3 α ,12 β -androstan-17-one, 3 α ,12 α -dihydroxy-5 β -androstan-17-one, 5 β -androstan-3 β ,12 β ,17 β -triol, 5 β -androstan-3 β ,12 α ,17 β -triol, 5 β -androstan-3 α ,12 β ,17 β -triol and 5 β -androstan-3 α ,12 α ,17 β -triol. The induction of 7 α -dehydroxylase and 12 α -dehydroxylase enzymes is discussed, together with the significance of dehydrogenation and ring fission under anaerobic conditions.

In studies reported in previous papers (Barnes *et al.*, 1976; Tenneson *et al.*, 1977, 1978a,b, 1979a,b) it has been shown that *Pseudomonas* sp. N.C.I.B. 10590 is capable of dehydrogenating the A ring of the bile acid nucleus and cleaving the side chain when grown under aerobic conditions.

Other workers have also shown that bacteria such as *Corynebacterium equi*, *Streptomyces gelaticus* (Hayakawa, 1973) and *Mycobacterium mucosum* (Severina *et al.*, 1968, 1969) are capable of cholic acid side-chain cleavage, resulting in the accumulation of C₂₂ 4-en-3-one and 4,6-dienone steroidal acids. Park (1981) has shown that *Pseudomonas* sp. A.T.C.C. 31752 grows aerobically on cattle bile, producing 12 β -hydroxyandrosta-4,6-diene-3,17-dione as a major product. This compound was probably derived from cholic acid after decon-

jugation of sodium taurocholate, the major bile salt in cattle bile.

The anaerobic degradation of cholic acid is less well documented, but it has been shown that *Escherichia coli* (Tenneson *et al.*, 1977) and *Bacteroides* spp. (Owen *et al.*, 1977) are capable of oxidizing the A ring and mediating side-chain cleavage, giving C₂₂ acidic and C₁₉ oxo steroids.

Minor transformations of cholic acid by anaerobic bacteria on the other hand have frequently been observed. The enzyme reactions that have been commonly reported are 7 α -dehydroxylation and hydroxy steroid dehydrogenation, these reactions being most easily demonstrated *in vitro* with bacteria that are common to the human intestinal tract. 7 α -Dehydroxylation of cholic acid is a predominant reaction carried out by intestinal bacteria, yielding deoxycholic acid as a major bile acid in faeces. Studies *in vitro* have revealed that this enzyme is induced in, among others, *Bacteroides* spp., *Clostridium* spp., *Veillonella* spp. (Hill & Drasar, 1968), and *Lactobacillus* spp. (Midtvedt & Norman, 1968).

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7 α -Hydroxy steroid dehydrogenation of cholic acid has been reported in many strains of *Escherichia coli* (Haslewood *et al.*, 1973; Aries & Hill, 1970), *Bacteroides fragilis* (Macdonald *et al.*, 1975; Aries & Hill, 1970), *Bacteroides* spp. (Hylemon & Stellwag, 1976) and *Clostridium perfringens* (Macdonald *et al.*, 1976).

3 α -Hydroxy steroid dehydrogenation has also been shown in *Clostridium perfringens* (Macdonald *et al.*, 1976) and *Eubacterium lentum* (Macdonald *et al.*, 1977). Furthermore *Clostridium* spp. have been shown to desaturate the cholic acid nucleus, giving 7 α ,12 α -dihydroxy-3-oxochol-4-en-24-oic acid (R. W. Owen, unpublished work). The high carriage rate of these clostridia correlates well with the incidence of colon cancer and forms the basis of a hypothesis described by Aries *et al.* (1969) implicating unsaturable bile acids in the aetiology of the disease.

This observation led us to study bile acid metabolism by bacteria under anaerobic conditions (simulating intestinal E_{H}), so that the extent of degradation and range of products could be delineated more fully.

In the present paper we describe the anaerobic metabolism of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590 and provide evidence for the structure and configuration of a number of acidic and neutral metabolites, of which the major unsaturated products have a 4,6-dienone structure.

Experimental

Materials

Cholic acid, 5 α -cholestane and androsta-1,4-diene-3,17-dione were obtained from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). General reagents were of AnalaR grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.). Solvents were redistilled immediately before use.

General methods

I.r., u.v., analysis by g.l.c. and t.l.c., and oxidation, acetylation and reduction on t.l.c. were performed as described previously (Tenneson *et al.*, 1979b). P.m.r. spectra were recorded on a JEOL 220 spectrometer operating at 220 MHz at 30°C from solutions in deuterated chloroform. Mass spectra were obtained with a Dupont 21-491 series mass spectrometer either by direct inlet or by combined g.l.c.-mass spectrometry with a Varian Aerograph 2700 gas chromatograph. Data reduction was performed with a Dupont 21-0948 data system.

Culture of *Pseudomonas* sp. N.C.I.B. 10590 in the presence of cholic acid

The biotransformation medium used in this study contained (g/l of water): sodium cholate, 1.0;

K₂HPO₄, 0.7; KH₂PO₄, 0.3; KNO₃, 1.0; MgSO₄·7H₂O, 0.1; FeSO₄·7H₂O, 0.0025; ZnSO₄·7H₂O, 0.0025; MnSO₄·4H₂O, 0.0025; final pH, 7.2. Solutions of sodium cholate, trace elements and MgSO₄ were autoclaved separately before mixing. Shake-flask cultures were grown on an LH Engineering orbital incubator at 28°C. Cells of *Pseudomonas* sp. N.C.I.B. 10590 obtained from four 1-litre aerobic shake-flask cultures (sodium cholate, 0.01%) by centrifugation at 10000g on an MSE Mistral 4L centrifuge were used to inoculate 4 litres of the culture medium. The cell pellet was taken up in 100 ml of culture medium and degassed with H₂/CO₂ (9:1) before inoculation. This gave a resting-cell suspension of the pseudomonad with a final cell density of 1×10^9 cells/ml ($A_{540} = 1.0$). The culture medium was steamed for 30 min and cooled immediately before inoculation to remove dissolved O₂. The top of the fermentation bottle was loosened (to allow gaseous exchange) before incubation at 28°C for 6 weeks under H₂/CO₂ (9:1) (with a palladium catalyst to remove residual O₂). The course of the fermentation and A_{540} (to monitor cell density) were followed at weekly intervals. Samples of the culture were extracted with ethyl acetate and examined by t.l.c. and g.l.c. for product formation. After 6 weeks' incubation the fermentation was terminated by direct extraction of the metabolites into an equal volume of ethyl acetate. After drying over anhydrous MgSO₄ the solvent was removed *in vacuo* at 30°C to yield 2.3 g of dried residue. The residue was separated into acidic (1.4 g) and neutral (926 mg) fractions. The acidic fraction was methylated with boron trifluoride/methanol and separated by preparative t.l.c. in methanol/dichloromethane (1:19, v/v), yielding residual cholic acid (120 mg) and a series of fractions from which steroids 19–22 were crystallized. The neutral fraction was separated likewise, giving steroids 2–18.

12 β -Hydroxyandrosta-4,6-diene-3,17-dione (compound 2, Fig. 1)

Recrystallization of compound 2 from methanol/dichloromethane gave white crystals (83 mg), m.p. 143–144°C. Characteristics: λ_{max} , 284 nm (ϵ 28000 M⁻¹·cm⁻¹); ν_{max} , 3545 (hydroxy), 1725 (17-ketone), 1664 (3-ketone), 1615 and 1580 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); δ 1.05, 1.15 (6H, s, 18-CH₃ and 19-CH₃), 2.34–2.62 (2H, m, 16-CH₂), 3.83 (1H, 4-line, m, $J = 5$ Hz, 10 Hz, 12-H), 5.72 (1H, s, 4-H) and 6.18 p.p.m. (2H, s, 6-H and 7-H); M^+ 300 (71%, C₁₉H₂₄O₃ requires M^+ 300), base peak at m/z 136 (4,6-dien-3-one, 100%) and low-intensity ion at m/z 282 ($M^+ - 18$, H₂O, 9%); g.l.c. R_F 2.10; t.l.c. R_F 0.91, after oxidation R_F 0.96, after acetylation R_F 1.02 and after reduction R_F 0.48.

12 α -Hydroxyandrosta-4,6-diene-3,17-dione (compound 3, Fig. 1)

Recrystallization of compound 3 from methanol/dichloromethane gave white spiky needles (46 mg), m.p. 178–180°C. Characteristics: $\lambda_{\max}$ 284 nm (ϵ 25 000 M⁻¹·cm⁻¹); $\nu_{\max}$ 2422 (hydroxy), 1728 (17-ketone), 1649 (3-ketone), 1618 and 1579 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); δ 0.95, 1.12 (6H, s, 18-CH₃ and 19-CH₃), 2.34–2.62 (2H, m, 16-CH₂), 4.18 (1H, t, J = 3 Hz, 12-H), 5.70 (1H, s, 4-H) and 6.19 p.p.m. (2H, s, 6-H and 7-H); M^+ 300 (22%, C₁₉H₂₄O₃ requires M^+ 300), base peak at m/z 136 (4,6-dien-3-one, 100%); and intense ion at m/z 282 (M^+ – 18, H₂O, 42%); g.l.c. R_F 2.20; t.l.c. R_F 0.75, after oxidation R_F 0.96, after acetylation R_F 1.02 and after reduction R_F 0.32.

12 β ,17 β -Dihydroxyandrosta-4,6-dien-3-one (compound 4, Fig. 1)

Recrystallization of compound 4 from methanol/dichloromethane gave white needles (35 mg), m.p. 130–132°C. Characteristics: $\lambda_{\max}$ 284 nm (ϵ 21 169 M⁻¹·cm⁻¹); $\nu_{\max}$ 3470 (hydroxy), 3340 (hydroxy), 1645 (3-ketone), 1608 and 1574 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); δ 0.91, 1.13 (6H, s, 18-CH₃ and 19-CH₃), 3.57 (1H, 4-line, m , J = 5 Hz, 10-Hz, 12-H), 3.94 (1H, t, J = 8 Hz, 17-H), 5.69 (1H, s, 4-H) and 6.09 p.p.m. (2H, s, 6-H and 7-H); M^+ 302 (38%, C₁₉H₂₆O₃ requires M^+ 302), intense ion at m/z 136 (4,6-dien-3-one, 80%) and low-intensity ions at m/z 284 (M^+ – 18, H₂O, 22%) and m/z 266 (M^+ – 36, 2H₂O, 12%); g.l.c. R_F 3.03; t.l.c. R_F 0.48, after oxidation R_F 0.96, after acetylation R_F 1.02 and after reduction R_F 0.48.

12 β -Hydroxyandrosta-1,4,6-triene-3,17-dione (compound 5, Fig. 1)

Recrystallization of compound 5 from methanol/dichloromethane gave white needles (8 mg), m.p. 226–228°C. Characteristics: $\lambda_{\max}$ 224, 257 and 300 nm (ϵ 13 100, 10 140 and 14 240 M⁻¹·cm⁻¹); $\nu_{\max}$ 3500 (hydroxy), 1735 (17-ketone), 1650 (3-ketone), 1620, 1600 and 1578 cm⁻¹ (C₍₁₎–C₍₂₎, C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); M^+ 298 (49%, C₁₉H₂₂O₃ requires M^+ 298), base peak at m/z 134 (1,4,6-trien-3-one, 100%) and low-intensity ion at m/z 280 (M^+ – 18, H₂O, 5%); g.l.c. R_F 2.31; t.l.c. R_F 0.70, after oxidation R_F 0.75, after acetylation R_F 1.01 and after reduction R_F 0.55.

12 β ,17 β -Dihydroxyandrosta-1,4,6-trien-3-one (compound 6, Fig. 1)

Recrystallization of compound 6 from methanol/dichloromethane gave white crystals (5 mg). Characteristics: $\lambda_{\max}$ 226, 256 and 298 nm (ϵ 11 270, 9870 and 13 450 M⁻¹·cm⁻¹); $\nu_{\max}$ 3420 (hydroxy), 3260 (hydroxy), 1650 (3-ketone), 1620, 1610 and

1585 cm⁻¹ (C₍₁₎–C₍₂₎, C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); M^+ 300 (22%, C₁₉H₂₄O₃ requires M^+ 300), base peak at m/z 134 (1,4,6-trien-3-one, 100%) and low-intensity ions at m/z 282 (M^+ – 18, H₂O, 11%) and m/z 264 (M^+ – 36, 2H₂O, 5%); g.l.c. R_F 3.20; t.l.c. R_F 0.55, after oxidation R_F 0.75, after acetylation R_F 0.96 and after reduction R_F 0.55.

12 α -Hydroxyandrosta-1,4-diene-3,17-dione (compound 7, Fig. 1)

Recrystallization of compound 7 from methanol/dichloromethane gave white spiky needles (7 mg), m.p. 222–223°C. Characteristics: $\lambda_{\max}$ 245 nm (ϵ 14 700 M⁻¹·cm⁻¹); $\nu_{\max}$ 3465 (hydroxy), 1736 (17-ketone), 1665 (3-ketone), 1622 and 1606 cm⁻¹ (C₍₁₎–C₍₂₎ and C₍₄₎–C₍₅₎ double bonds); M^+ 300 (10%, C₁₉H₂₄O₃ requires M^+ 300), base peak at m/z 121 (1,4-dien-3-one, 100%) and intense ion at m/z 282 (M^+ – 18, H₂O, 99%); g.l.c. R_F 2.50; t.l.c. R_F 0.54, after oxidation R_F 0.85, after acetylation R_F 0.99 and after reduction R_F 0.14.

12 β -Hydroxy-5 β -androstane-3,17-dione (compound 8, Fig. 1)

Recrystallization of compound 8 from methanol/dichloromethane gave white crystals (94 mg), m.p. 149–150°C. Characteristics: $\lambda_{\max}$ 286 nm (ϵ 212 M⁻¹·cm⁻¹); $\nu_{\max}$ 3540 (hydroxy) and 1718 cm⁻¹ (17-ketone and 3-ketone); δ 0.96, 1.06 (6H, s, 18-CH₃ and 19-CH₃), 2.12–2.34 (2H, m, 16-CH₂), 3.04 (1H, s, OH) and 3.85 p.p.m. (1H, 4-line, m , J = 5 Hz, 10-Hz, 12-H); M^+ 304 (100%, C₁₉H₁₈O₃ requires M^+ 304) and low-intensity ions at m/z 289 (M^+ – 15, CH₃, 4%) and m/z 286 (M^+ – 18, H₂O, 5%); g.l.c. R_F 1.40; t.l.c. R_F 0.90, after oxidation R_F 0.94, after acetylation R_F 1.16 and after reduction R_F 0.20.

12 α -Hydroxy-5 β -androstane-3,17-dione (compound 9, Fig. 1)

Recrystallization of compound 9 from methanol/dichloromethane gave white crystals (47 mg), m.p. 159–160°C. Characteristics: $\lambda_{\max}$ 285 nm (ϵ 180 M⁻¹·cm⁻¹); $\nu_{\max}$ 3495 (hydroxy), 1720 (17-ketone), and 1708 cm⁻¹ (3-ketone); δ 0.87, 1.02 (6H, s, 18-CH₃ and 19-CH₃), 2.26–2.55 (2H, m, 16-CH₂) and 4.15 p.p.m. (1H, t, J = 3 Hz, 12-H); M^+ 304 (100%, C₁₉H₂₆O₃ requires M^+ 304), low-intensity ion at m/z 289 (M^+ – 15, CH₃, 14%) and intense ion at m/z 286 (M^+ – 18, H₂O, 36%); g.l.c. R_F 1.37; t.l.c. R_F 0.74, after oxidation R_F 0.94, after acetylation R_F 1.15 and after reduction R_F 0.16.

3 β ,12 β -Dihydroxy-5 β -androstan-17-one (compound 10, Fig. 2)

Recrystallization of compound 10 from methanol/dichloromethane gave white crystals

(102 mg). Characteristics: $\lambda_{\max}$ 292 nm (ϵ 120 M⁻¹·cm⁻¹); $\nu_{\max}$ 3505 (hydroxy), 3420 (hydroxy) and 1725 cm⁻¹ (17-ketone); δ 0.93, 0.96 (6H, s, 18-CH₃ and 19-CH₃), 2.38–2.56 (2H, m, 16-CH₂), 3.64 (1H, m, 3-H) and 3.79 p.p.m. (1H, 4-line, m, $J = 5$ Hz, 10 Hz, 12-H); M^+ 306 (87%, C₁₉H₃₀O₃ requires M^+ 306), intense ion at m/z 288 ($M^+ - 18$, H₂O, 62%) and low-intensity ion at m/z 270 ($M^+ - 36$, 2H₂O, 22%); g.l.c. R_F 1.20; t.l.c. R_F 0.50, after oxidation R_F 0.94, after acetylation R_F 1.20 and after reduction R_F 0.20.

3 β ,12 α -Dihydroxy-5 β -androstan-17-one (compound 11, Fig. 1)

Recrystallization of compound 11 from methanol/dichloromethane gave white crystals (25 mg), m.p. 168°C. Characteristics: $\lambda_{\max}$ 286 nm (ϵ 90 M⁻¹·cm⁻¹); $\nu_{\max}$ 3505 (hydroxy), 3420 (hydroxy) and 1732 cm⁻¹ (17-ketone); M^+ 306 (95%, C₁₉H₃₀O₃ requires M^+ 306), base peak at m/z 288 ($M^+ - 18$, H₂O, 100%) and low-intensity ion at m/z 270 ($M^+ - 36$, 2H₂O, 17%); g.l.c. R_F 1.25; t.l.c. R_F 0.48, after oxidation R_F 0.94, after acetylation R_F 1.24 and after reduction R_F 0.16.

3 α ,12 β -Dihydroxy-5 β -androstan-17-one (compound 12, Fig. 2)

Recrystallization of compound 12 from methanol/dichloromethane gave white crystals (6 mg). Characteristics: $\lambda_{\max}$ 288 nm (ϵ 160 M⁻¹·cm⁻¹); $\nu_{\max}$ 3485 (hydroxy), 3405 (hydroxy) and 1735 cm⁻¹ (17-ketone); M^+ 306 (100%, C₁₉H₃₀O₃ requires M^+ 306), intense ion at m/z 288 ($M^+ - 18$, H₂O, 52%) and m/z 270 (M^+ , 2H₂O, 73%); g.l.c. R_F 1.10; t.l.c. R_F 0.43, after oxidation R_F 0.94, after acetylation R_F 1.28 and after reduction R_F 0.14.

3 α ,12 α -Dihydroxy-5 β -androstan-17-one (compound 13, Fig. 2)

Recrystallization of compound 13 from methanol/dichloromethane gave white crystals (4 mg), m.p. 124°C. Characteristics: $\lambda_{\max}$ 295 nm (ϵ 20 M⁻¹·cm⁻¹); $\nu_{\max}$ 3490 (hydroxy), 3435 (hydroxy) and 1735 cm⁻¹ (17-ketone); M^+ 306 (12%, C₁₉H₃₀O₃ requires M^+ 306), low-intensity ion at m/z 288 ($M^+ - 18$, H₂O, 41%) and base peak at m/z 270 ($M^+ - 36$, 2H₂O, 100%); g.l.c. R_F 1.18; t.l.c. R_F 0.36, after oxidation R_F 0.94, after acetylation R_F 1.24 and after reduction R_F 0.12.

5 β -Androstane-3 β ,12 β ,17 β -triol (compound 14, Fig. 2)

Recrystallization of compound 14 from methanol/dichloromethane gave white crystals (1 mg). Characteristics: $\nu_{\max}$ 3450 cm⁻¹ (hydroxy groups, broad); g.l.c. R_F 1.70; t.l.c. R_F 0.20, after oxidation R_F 0.94, after acetylation R_F 1.11 and after reduction R_F 0.20.

5 β -Androstane-3 β ,12 α ,17 β -triol (compound 15, Fig. 2)

Recrystallization of compound 15 from methanol/dichloromethane gave white crystals (1 mg). Characteristics: $\nu_{\max}$ 3430 cm⁻¹ (hydroxy groups, broad); g.l.c. R_F 1.70; t.l.c. R_F 0.16, after oxidation R_F 0.94, after acetylation R_F 1.13 and after reduction R_F 0.16.

5 β -Androstane-3 α ,12 β ,17 β -triol (compound 16, Fig. 2)

Recrystallization of compound 16 from methanol/dichloromethane gave white crystals (3 mg), m.p. 160°C. Characteristics: $\nu_{\max}$ 3450 cm⁻¹ (hydroxy groups, broad); g.l.c. R_F 1.50; t.l.c. R_F 0.14, after oxidation R_F 0.94, after acetylation R_F 1.18 and after reduction R_F 0.14.

5 β -Androstane-3 α ,13 α ,17 β -triol (compound 17, Fig. 2)

Recrystallization of compound 17 from methanol/dichloromethane gave white crystals (4 mg). Characteristics: $\nu_{\max}$ 3465 cm⁻¹ (hydroxy groups, broad), M^+ (no detectable M^+ typical of trihydroxyandrostanes), low-intensity ions at m/z 290 ($M^+ - 18$, H₂O, 23%), at m/z 272 ($M^+ - 36$, 2H₂O, 17%) and at m/z 254 ($M^+ - 54$, 3H₂O, 5%); g.l.c. R_F 1.40; t.l.c. R_F 0.12, after oxidation R_F 0.94, after acetylation R_F 1.16 and after reduction R_F 0.12.

3-Hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (compound 18, Fig. 2)

Recrystallization of compound 18 from methanol/dichloromethane gave yellow crystals (6 mg), m.p. 122°C. Characteristics: $\lambda_{\max}$ 218 nm and 283 nm; $\nu_{\max}$ 3360 (hydroxy), 1740 (17-ketone), 1705 (9-ketone), 1608, 1585, 1500 (aromatic ring), 1280, 1250 (phenol), 855 and 800 cm⁻¹ (*o,m*-substituted phenol ring); M^+ 300 (39%, C₁₉H₂₄O₃ requires M^+ 300), base peak at m/z 134 (phenolic A ring + 2C fragment, 100%) and intense ion at m/z 122 (phenolic A ring, 63%), M^+ (trimethylsilyl ether), 372 (36%), base peak at m/z 206 (phenolic A ring + 2C fragment + Me₃Si, 100%) and low-intensity ion at m/z 194 (trimethylsilyl phenolic A ring, 25%); g.l.c. R_F 1.24; t.l.c. R_F 0.86, after oxidation R_F 0.86, after acetylation R_F 1.22 and after reduction R_F 0.44.

Methyl 12 α -hydroxy-3-oxochola-4,6-dien-24-oate (methyl ester of compound 19, Fig. 2)

Recrystallization of the methyl ester of compound 19 from diethyl ether gave white spiky needles (200 mg), m.p. 144–146°C. Characteristics: $\lambda_{\max}$ 284 nm (ϵ 28 000 M⁻¹·cm⁻¹); $\nu_{\max}$ 3505 (hydroxy), 1719 (carboxy), 1664 (3-ketone), 1612 and 1578 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); δ

0.79, 1.10 (6H, s, 18-CH₃ and 19-CH₃), 0.99 (3H, d, $J = 6$ Hz, 21-CH₃), 3.68 (3H, s, 24-OCH₃), 5.08 (1H, t, $J = 3$ Hz, 12-H), 5.68 (1H, s, 4-H) and 6.12 (2H, s, 6-H and 7-H); M^+ 400 (6%, C₂₅H₃₆O₄ requires M^+ 400), base peak at m/z 267 ($M^+ - 133$, 6C side chain + H₂O, 100%), and low-intensity ions at m/z 136 (4,6-dien-3-one, 11%) and m/z 382 ($M^+ - 18$, H₂O, 34%); g.l.c. R_F 13.20; t.l.c. R_F 0.83, after oxidation R_F 1.08, after acetylation R_F 1.11 and after reduction R_F 0.83.

Methyl 3,12-dioxochola-4,6-dien-24-oate (methyl ester of compound 20, Fig. 3)

Recrystallization of the methyl ester of compound 20 from diethyl ether gave white needles (55 mg), m.p. 139–140°C. Characteristics: $\lambda_{\max}$ 284 nm (ϵ 22 400 M⁻¹·cm⁻¹); $\nu_{\max}$ 1720 (carboxy), 1705 (12-ketone), 1653 (3-ketone), 1612 and 1580 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); δ 0.88, 1.20 (6H, s, 18-CH₃ and 19-CH₃), 1.13 (3H, s, 21-CH₃), 3.68 (3H, s, 24-OCH₃), 5.73 (1H, s, 4-H) and 6.14 p.p.m. (2H, s, 6-H and 7-H); M^+ 398 (100%, C₂₅H₃₄O₄ requires M^+ 398) and low-intensity ions at m/z 136 (4,6-dien-3-one, 33%) and at m/z 283 ($M^+ - 115$, 6C side chain, 34%); g.l.c. R_F 12.0; t.l.c. R_F 1.08, after oxidation R_F 1.08, after acetylation R_F 1.08 and after reduction R_F 0.85.

Methyl 12 α -hydroxy-3-oxopregna-4,6-diene-20-carboxylate (methyl ester of compound 21, Fig. 3)

Recrystallization of the methyl ester of compound 21 from diethyl ether gave a slightly impure crystalline mixture (30 mg). Characteristics: $\lambda_{\max}$ 284 nm (ϵ 24 620 M⁻¹·cm⁻¹); $\nu_{\max}$ 3505 (hydroxy), 1746 (carboxy), 1666 (3-ketone), 1618 and 1586 cm⁻¹ (C₍₄₎–C₍₅₎ and C₍₆₎–C₍₇₎ double bonds); M^+ (trimethylsilyl ether) 444 [10%, C₂₃H₃₂O₄ + Me₃Si (72) requires M^+ 444], low-intensity ion at m/z 354 ($M^+ - 90$, 12-trimethylsilyl ether, 5%) and at m/z 267 ($M^+ - 177$, 4C side chain + 12-trimethylsilyl ether, 21%); g.l.c. R_F 5.80; t.l.c. R_F 0.64, after oxidation R_F 1.08, after acetylation R_F 1.08 and after reduction R_F 0.64.

Methyl 7 α ,12 α -dihydroxy-3-oxo-5 β -cholan-24-oate (methyl ester of compound 22, Fig. 3)

Recrystallization of the methyl ester of compound 22 from diethyl ether gave white crystals (170 mg), m.p. 172°C. Characteristics: $\lambda_{\max}$ 282 nm (ϵ 271 M⁻¹·cm⁻¹); $\nu_{\max}$ 3500 (hydroxy), 3450 (hydroxy), 1736–1720 (carboxy) and 1700 cm⁻¹ (3-ketone); δ 0.67, 0.96 (6H, s, 18-CH₃ and 19-CH₃), 1.21 (3H, d, $J = 6$ Hz, 21-CH₃), 3.66 (3H, s, 24-OCH₃), 4.0 (1H, s, 12-H) and 4.05 p.p.m. (1H, s, 7-H); M^+ 420 (13%, C₂₅H₄₀O₅ requires M^+ 420), base peak at m/z 287 ($M^+ - 133$, 6C side chain + H₂O, 100%) and low-intensity ions at m/e 402 ($M^+ - 18$, H₂O, 42%), m/z 284 ($M^+ - 36$,

2H₂O, 20%) and m/z 269 ($M^+ - 151$, 6C side chain + 2H₂O, 93%); g.l.c. R_F 13.95; t.l.c. R_F 0.31, after oxidation R_F 1.05, after acetylation R_F 1.07 and after reduction R_F 0.18.

Results

A resting-cell suspension of *Pseudomonas* sp. N.C.I.B. 10590 was found to be capable of degrading cholic acid, giving rise to a range of steroidal products. Twenty-one metabolites (17 neutral and four acidic) were isolated in the yields indicated in Table 1 and the assigned structures are depicted in Figs. 1–3. The neutral compounds can be divided into two distinct groups, namely C₁₉ unsaturated androstanes, which first appeared during the second week (reaching a maximum during the fourth week) of the fermentation; and C₁₉ saturated androstanes. The structure elucidation of the unsaturated androstanes is discussed first.

The major neutral compound (2) of the unsaturated androstane group showed a molecular ion at m/z 300 and an intense ion (base peak) at m/z 136 in the mass spectrometer, suggesting a steroidal 4,6-dien-3-one AB-ring structure (Zaretskii, 1976). Confirmation of this structure was provided by the i.r. spectrum (1664, 1615 and 1580 cm⁻¹, $\alpha\beta$ -unsaturated ketone), by the u.v. spectrum [$\lambda_{\max}$ 284 nm, di- β -substituted $\alpha\beta$ -unsaturated ketone in a six-membered ring, double bond exocyclic, extended by a double bond at C₍₆₎–C₍₇₎ (Dorfman, 1953)] and by the p.m.r. spectrum (three vinylic protons in the range δ 5.72–6.18 p.p.m.). Compound 2 was easily oxidized, acetylated and reduced, suggesting the presence of both a hydroxy and an unconjugated ketone group. This was confirmed by the i.r. spectrum, which displayed a peak at 3545 cm⁻¹ characteristic of a hydroxy group and a peak at 1725 cm⁻¹ characteristic of an unconjugated ketone group. The p.m.r. spectrum of compound 2 gave rise to a four-line pattern centred at δ 3.83 p.p.m. (1H, m, $J = 5$ Hz and 10 Hz), the proton at C₍₁₂₎ causing spin-spin coupling with the α - and β -protons at C₍₁₁₎. The dihedral (Karplus) angles for the 12 α -proton are about 60° and 180°, giving two coupling constants at 5 Hz and 10 Hz. The ketone group at C₍₁₇₎ causes a deshielding effect, increasing the expected chemical shift from 3.45 to 3.83 p.p.m. on the δ scale. On this basis the hydroxy group in compound 2 was assigned the 12 β -configuration. Further support for the assignment of the configuration of the hydroxy group is provided by the observation (Zeitz & Spiteller, 1974) that loss of the elements of water from the molecular ion in the mass spectrometer occurs more readily with axial hydroxy groups (usually giving rise to an ion of higher relative intensity than the M^+) than with β -equatorial groups (usually giving rise to an ion of lower relative

Table 1. Yield of metabolites with respect to cholic acid starting material after 6 weeks' anaerobic incubation of *Pseudomonas* sp. N.C.I.B. 10590

Experimental details are given in the text.

Metabolite no.	Identification	Yield (%)
2	12 β -Hydroxyandrosta-4,6-diene-3,17-dione	1.54
3	12 α -Hydroxyandrosta-4,6-diene-3,17-dione	0.85
4	12 β ,17 β -Dihydroxyandrosta-4,6-dien-3-one	0.65
5	12 β -Hydroxyandrosta-1,4,6-triene-3,17-dione	0.15
6	12 β ,17 β -Dihydroxyandrosta-1,4,6-triene-3-one	0.09
7	12 α -Hydroxyandrosta-1,4-diene-3,17-dione	0.13
8	12 β -Hydroxy-5 β -androstane-3,17-dione	1.76
9	12 α -Hydroxy-5 β -androstane-3,17-dione	0.88
10	3 β ,12 β -Dihydroxy-5 β -androstan-17-one	1.91
11	3 β ,12 α -Dihydroxy-5 β -androstan-17-one	0.47
12	3 α ,12 β -Dihydroxy-5 β -androstan-17-one	0.11
13	3 α ,12 α -Dihydroxy-5 β -androstan-17-one	0.08
14	5 β -Androstane-3 β ,12 β ,17 β -triol	0.02
15	5 β -Androstane-3 β ,12 α ,17 β -triol	0.02
16	5 β -Androstane-3 α ,12 β ,17 β -triol	0.06
17	5 β -Androstane-3 α ,12 α ,17 β -triol	0.08
18	3-Hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione	0.11
19	12 α -Hydroxy-3-oxochola-4,6-dien-24-oic acid	4.75
20	3,12-Dioxochola-4,6-dien-24-oic acid	1.29
21	12 α -Hydroxy-3-oxopregna-4,6-diene-20-carboxylic acid	0.66
22	7 α ,12 α -Dihydroxy-3-oxo-5 β -cholan-24-oic acid	4.21

intensity than the M^+). With compound 2 a low-intensity ion at m/z 282 (9%) was observed, corresponding to the loss of a β -equatorial group from the molecular ion (71%). Compound 2 has therefore been assigned the structure 12 β -hydroxyandrosta-4,6-diene-3,17-dione.

One of the minor neutral compounds (3) of the unsaturated androstane group showed a molecular ion also at m/z 300 and an intense ion (base peak) at m/z 136 in the mass spectrometer, indicative of a 4,6-dienone AB-ring structure. Confirmation of this structure was provided by the i.r., u.v. and p.m.r. spectra. Compound 3 was easily oxidized, acetylated and reduced, suggesting the presence of a hydroxy group and a unconjugated ketone group, and this was confirmed by the i.r. spectrum. The p.m.r. spectrum of compound 3 displayed a triplet at δ 4.18 p.p.m. The β -proton bisects the dihedral angle between the adjacent α - and β -C₍₁₁₎ protons, and only one coupling constant ($J = 3$ Hz) is observed. The C₍₁₇₎ ketone group again caused an increase of the expected chemical shift on the δ scale. On this basis the hydroxy group in compound 3 was assigned the 12 α -configuration. An intense ion (with respect to the molecular ion) at m/z 282 (42%) was observed, corresponding to the loss of a α -axial hydroxy group from the molecular ion (22%), confirmed the assignment. Compound 3 was therefore assigned the structure 12 α -hydroxyandrosta-4,6-diene-3,17-dione.

Another of the minor neutral compounds (4) in the unsaturated androstane group showed a

molecular ion at m/z 302 and an intense ion at m/z 136, suggesting a steroidal 4,6-dien-3-one AB-ring structure. Confirmation of this structure was provided by the i.r., u.v. and p.m.r. spectra. Compound 4 was easily oxidized and acetylated, but could not be reduced, suggesting the presence of hydroxy groups and the absence of unconjugated ketone groups. This was confirmed by the i.r. spectrum. The p.m.r. spectrum of compound 4, like that of compound 2, displayed a four-line pattern, but this time closer (δ 3.57 p.p.m.) to the expected value of δ 3.4 p.p.m. (for a 12 α -proton). As well as this pattern, a well-defined triplet (centred at δ 3.94 p.p.m.) at the slightly higher than expected value of δ 3.82 p.p.m. (for a 17 α -proton) was observed. In this case the 17 α -proton presumably bisects the dihedral angle between the two protons at C₍₁₆₎, with one coupling constant of 8 Hz in evidence. The presence of a 12 β -hydroxy group causes an increase in the expected chemical shift. On this basis the hydroxy groups in compound 4 were assigned the 12 β - and 17 β -configurations. Low-intensity ions at m/z 284 (22%) and m/z 266 (12%) were observed in the mass spectrum, corresponding to the loss of equatorial 12 β - and 17 β -hydroxy groups from the molecular ion (38%), confirm the assignment of β -configuration designated to the hydroxy groups at C₍₁₂₎ and C₍₁₇₎. Compound 4 was therefore assigned the structure 12 β ,17 β -dihydroxyandrosta-4,6-dien-3-one.

Of the three remaining unsaturated metabolites, compound 5 was identical in its t.l.c., g.l.c. and

Fig. 1. Neutral metabolites isolated after the anaerobic degradation of cholic acid (I) by *Pseudomonas* sp. N.C.I.B. 10590

spectroscopic properties with 12 β -hydroxy-androsta-1,4,6-triene-3,17-dione (Tenneson *et al.*, 1979c).

The mass spectrum of compound 6 showed a molecular ion at m/z 300 and an intense ion (base peak) at m/z 134 typical of a steroidal 1,4,6-trienone structure (Zaretskii, 1976). Confirmation of this A/B-ring structure was provided by the u.v. spectrum (λ_{max} , 224, 256 and 300 nm) and by the i.r. spectrum. Compound 6 was easily oxidized to a product that corresponded in R_{ADD} (mobility relative to that of androsta-1,4-diene-3,17-dione) and

colour (mauve) on t.l.c. to the oxidation product of compound 5. Compound 6 resisted reduction, but was easily acetylated, indicating the presence of hydroxy groups and the absence of unconjugated ketone groups. This was confirmed by the i.r. spectrum. Sufficient compound 6 was not available for p.m.r. analysis, and so the position and stereochemistry of the hydroxy groups was inferred from the mass spectrum. The mass spectrum showed low-intensity ions at m/z 282 (11%) and m/z 264 (5%) due to the loss of β -equatorial hydroxy groups from the molecular ion (22%). Support for the assigned position and stereochemistry of the hydroxy groups comes from the fact that the reduction product of compound 5 was identical in colour and R_{ADD} with compound 6 on t.l.c. Compound 6 has therefore been assigned the structure 12 β ,17 β -dihydroxyandrosta-1,4,6-trien-3-one.

The mass spectrum of compound 7 showed a molecular ion at m/z 300 and an intense ion (base peak) at m/z 121, suggesting a steroidal 1,4-dienone A-ring structure (Budzikiewicz, 1972). Confirmation of this structure was provided by the i.r. spectrum (1665, 1622 and 1606 cm^{-1} , $\alpha\beta$ -unsaturated ketone) and by the u.v. spectrum [λ_{max} , 245 nm, di- β -substituted $\alpha\beta$ -unsaturated ketone in a six-membered ring, double bond exocyclic (Dorfman, 1953)]. Compound 7 was easily oxidized, acetylated and reduced, indicating the presence of both a hydroxy group and an unconjugated ketone group. This was confirmed by the i.r. spectrum. The mass spectrum contained an intense ion at m/z 282 (99%) due to the loss of the elements of water from the molecular ion (10%). Compound 7 was therefore identical with 12 α -hydroxyandrosta-1,4-diene-3,17-dione described by Barnes *et al.* (1976).

Three major and seven minor saturated C₁₉ androstane compounds were isolated. Two of the major compounds in this group, compounds 8 and 9, appeared from their mass-spectral behaviour to be isomers of each other. The mass spectra of both gave many peaks characteristic of monohydroxy saturated androstanes (Zeitz & Spiteller, 1974). Both compounds gave identical base-peak molecular ions at m/z 304. Compound 8 also gave the following characteristic major ions at m/z 55 (59%), m/z 67 (34%), m/z 81 (35%), m/z 95 (31%), m/z 109 (31%), m/z 121 (29%), m/z 135 (19%), m/z 147 (22%), m/z 244 (10%), m/z 247 (27%), m/z 260 (30%) and m/z 286 (6%, $M^+ - \text{H}_2\text{O}$), which is identical with the spectrum published by Zeitz & Spiteller (1974) for 12 β -hydroxy-5 β -androstane-3,17-dione produced by chemical synthesis. Compound 9 also gave the following characteristic major ions at m/z 55 (99%), m/z 67 (42%), m/z 81 (47%), m/z 97 (60%), m/z 109 (35%), m/z 121 (31%), m/z 135 (22%), m/z 147 (23%), m/z 244 (22%), m/z 247 (27%), m/z 260 (76%), m/z 286

Compound 10

Compound 11

Compound 12

Compound 13

Compound 14

Compound 15

Compound 16

Compound 17

Compound 18

Compound 23

Fig. 2. Neutral metabolites isolated after the anaerobic degradation of cholic acid (1) by *Pseudomonas* sp. N.C.I.B. 10590

Fig. 3. Acidic metabolites isolated after the anaerobic degradation of cholic acid (1) by *Pseudomonas* sp. N.C.I.B. 10590

(68%, $M^+ - H_2O$) and m/z 289 (22%, $M^+ - CH_3$), which is almost identical with the spectrum of 12 α -hydroxy-5 β -androstane-3,17-dione (Zeitz & Spiteller, 1974). Confirmation of the saturated nature of these compounds was provided by the i.r. spectra, which did not display any evidence for $\alpha\beta$ -unsaturated ketones, and the p.m.r. spectra, which did not show the presence of any vinylic protons.

The oxidation products of compounds 8 and 9 were identical in R_{ADD} and colour (rust) on t.l.c. with the known trione, thus showing that the oxygen substitution pattern was the same for both compounds. Both compounds were easily acetylated and reduced, suggesting the possession of a hydroxy group and an unconjugated ketone group. The presence of a hydroxy group was confirmed by the i.r. spectra, which displayed peaks in the hydroxy group region, and two significant peaks in the

carbonyl region indicated the presence of two and not one unconjugated ketone groups.

The position and stereochemistry of the hydroxy groups on compounds 8 and 9 were elucidated by p.m.r. The p.m.r. spectrum of compound 8 gave rise to a four-line pattern centred at δ 3.85 p.p.m. (1 H, m , $J = 5$ Hz and 10 Hz), indicating the presence of a 12 β -hydroxy group. The p.m.r. spectrum of compound 9 displayed a well defined triplet centred at δ 4.15 p.p.m. ($J = 3$ Hz), indicating the presence of a 12 α -hydroxy group. Compounds 8 and 9 have therefore been assigned the structures 12 β -hydroxy-5 β -androstane-3,17-dione and 12 α -hydroxy-5 β -androstane-3,17-dione respectively.

The other major saturated androstane compound (10), along with three of the minor components (compounds 11–13), appeared from their t.l.c., g.l.c. and mass-spectral behaviour to be closely related compounds. The mass spectra of all four gave many

peaks characteristic of dihydroxy saturated androstanes (Zeitz & Spiteller, 1974). The four compounds all gave identical molecular ions at m/z 306 (compound 11, M^+ 306, 87%; compound 12, M^+ 306, 95%; compound 13, M^+ 306, 100%; compound 14, M^+ 306, 11%), suggesting that they were closely related to compounds 8 and 9.

The mass spectrum of compound 10 contained the following characteristic major ions at m/z 41 (100%), m/z 55 (96%), m/z 67 (56%), m/z 81 (60%), m/z 97 (98%), m/z 107 (55%), m/z 121 (46%), m/z 133 (24%), m/z 147 (30%), m/z 201 (20%), m/z 232 (31%), m/z 262 (17%), m/z 270 (22%, $M^+ - 36$, $2H_2O$), m/z 273 (28%) and m/z 288 (62%, $M^+ - 18$, H_2O), which is very similar to that of 3 β ,12 β -dihydroxy-5 β -androstan-17-one (Zeitz & Spiteller, 1974).

The mass spectrum of compound 11 contained the following characteristic major ions at m/z 55 (26%), m/z 67 (20%), m/z 81 (34%), m/z 97 (83%), m/z 107 (42%), m/z 121 (44%), m/z 133 (20%), m/z 147 (28%), m/z 160 (20%), m/z 201 (18%), m/z 232 (29%), m/z 262 (25%), m/z 270 (18%, $M^+ - 36$, $2H_2O$), m/z 273 (32%) and m/z 288 (100%, $M^+ - 18$, H_2O), which is very similar to that of 3 β ,12 α -dihydroxy-5 α -androstan-17-one (Zeitz & Spiteller, 1974).

The mass spectrum of compound 12 contained the following characteristic major ions at m/z 55 (21%), m/z 81 (29%), m/z 97 (51%), m/z 107 (38%), m/z 121 (36%), m/z 133 (19%), m/z 147 (37%), m/z 161 (17%), m/z 201 (13%), m/z 232 (17%), m/z 262 (31%), m/z 270 (73%, $M^+ - 36$, $2H_2O$), m/z 273 (23%) and m/z 288 (53%, $M^+ - 18$, H_2O).

The mass spectrum of compound 13 contained the following characteristic major ions at m/z 55 (20%), m/z 81 (24%), m/z 97 (31%), m/z 107 (28%), m/z 121 (25%), m/z 133 (15%), m/z 147 (33%), m/z 174 (18%), m/z 216 (29%), m/z 242 (12%), m/z 252 (17%), m/z 255 (32%), m/z 260 (48%), m/z 270 (100%, $M^+ - 36$, $2H_2O$) and m/z 288 (56%, $M^+ - 18$, H_2O), which is almost identical with that of 3 α ,12 α -dihydroxy-5 β -androstan-17-one (Zeitz & Spiteller, 1974).

Confirmation of the saturated nature of these compounds was provided by the i.r. spectra, which did not display any evidence for $\alpha\beta$ -unsaturated ketones, and in the case of compound 10 by the p.m.r. spectrum, which did not show any vinylic protons.

The oxidation products of compounds 10–13 were identical in R_{ADD} and colour (rust) on t.l.c. to the oxidation product of compounds 8 and 9, thus showing that the oxygen substitution pattern was the same in all four compounds. All four compounds were acetylated and reduced on t.l.c., indicating the presence of hydroxy groups and an unconjugated ketone group. This was confirmed by the i.r. spectra,

which gave two peaks in the hydroxy-group region and one peak in the carbonyl-group region.

The position and stereochemistry of the hydroxy groups in compound 10 were elucidated from the p.m.r. spectrum. Insufficient material was available to take p.m.r. spectra of compounds 11–13, and so the position and stereochemistry of the hydroxy groups have been inferred from the mass spectra.

Compound 10 gave rise to a four-line pattern in the p.m.r. spectrum centred at δ 3.79 p.p.m. (1H, m, $J = 5$ Hz and 10 Hz). The ketone group at C₍₁₇₎ caused a deshielding effect, increasing the expected chemical shift from δ 3.45 p.p.m. to δ 3.79 p.p.m. The spectrum also contained a broad multiplet centred at δ 3.64 p.p.m. at the expected value for a 3 α -proton (Bridgeman *et al.*, 1970). On this basis the hydroxy groups on compound 10 were assigned the 3 β - and 12 β -configuration.

Without the aid of p.m.r. spectra, the position and stereochemistry of the hydroxy groups in compounds 11–13 were more difficult to deduce. The i.r. spectra, in comparison with known standards, gave peaks in the carbonyl region corresponding very closely to 17-C=O (1720 cm⁻¹) groups, which differed significantly from 12-C=O (1700 cm⁻¹) and 3-C=O (1710 cm⁻¹) groups. However, the mass spectra gave the greatest indication of the substituent patterns. The spectra of all three plus that of compound 10 gave a key ion at m/z 97 specific to 17-oxo steroids (Zeitz & Spiteller, 1974) carrying a hydroxy group at C₍₁₂₎. The mass spectra therefore show conclusively that compounds 11–13, like compound 10, are dihydroxy-17-oxoandrostanes. Having established that the ketone group was at C₍₁₇₎, the hydroxy groups must therefore, unless transhydroxylation had occurred (which seems unlikely), have been at C₍₃₎ and C₍₁₂₎. It is also possible to assign the stereochemistry of the hydroxy groups from the mass spectra, taking into account the observation by Zeitz & Spiteller (1974) that the elimination of α -axial hydroxy groups occurs more readily than with β -equatorial groups, in conjunction with the observation by Dean & Alpin (1966) that 12-hydroxy groups are eliminated before 3-hydroxy groups in the mass spectrometer.

The mass spectrum of compound 11 revealed that $M^+ - 18$ was more intense than $M^+ - 36$. Indeed, it gave rise to a more intense ion than the M^+ . Thus it can be deemed that $M^+ - 18$ (100%) is due to the loss of a 12 α -hydroxy group and that $M^+ - 36$ (17%) is due to the additional loss of a 3 β -hydroxy group from the molecular ion (95%). Compound 11 has therefore been assigned the structure 3 β ,12 α -dihydroxy-5 β -androstan-17-one.

The mass spectrum of compound 12 revealed that $M^+ - 18$ (52%) was less intense than $M^+ - 36$ (73%) and the M^+ (100%). Thus it can be deemed that $M^+ - 18$ is due to the loss of a 12 β -hydroxy group

and that $M^+ - 36$ is due to the additional loss of a 3 α -hydroxy group. Compound 12 has therefore been assigned the structure 3 α ,12 β -dihydroxy-5 β -androstan-17-one.

The mass spectrum of compound 13 revealed that $M^+ - 18$ (56%) was less intense than $M^+ - 36$ (100%). However, both ions were of far greater intensity than the M^+ (11%), indicating the ready loss of water in both cases. Thus it can be deduced that $M^+ - 18$ is due to the loss of a 12 α -hydroxy group and that $M^+ - 36$ is due to the loss of a 3 α -hydroxy group. Compound 13 has therefore been assigned the structure 3 α ,12 α -dihydroxy-5 β -androstan-17-one.

Four other saturated androstane compounds (14–17) were isolated in very small amounts. All four compounds could not be reduced, but were easily acetylated, indicating the presence of hydroxy groups and the absence of an unconjugated ketone group. The oxidation product of the compounds was identical with that of compounds 8 and 9, indicating that the substitution pattern was the same in all four. Furthermore, the reduction products of compounds 10, 11, 12 and 13 were identical in colour and R_{ADD} on t.l.c. with compounds 14, 15, 16 and 17 respectively. This indicated that compounds 14–17 were the fully reduced analogues of the dihydroxy androstane compounds 10–13. This was confirmed by the i.r. spectra, which displayed peaks in the hydroxy-group region with none in the carbonyl group region.

The mass spectrum of compound 17 gave the following major ions typical of trihydroxyandrostanes (Zeitz & Spiteller, 1974) at m/z 55 (53%), m/z 67 (29%), m/z 81 (71%), m/z 93 (32%), m/z 95 (30%), m/z 107 (31%), m/z 147 (27%), m/z 163 (47%), m/z 257 (99%), m/z 272 (17%, $M^+ - 36$, $2\text{H}_2\text{O}$), m/z 275 (8%) and m/z 290 (23%, $M^+ - 18$, H_2O). The spectrum is notable for the absence of a molecular ion at m/z 308. However, this is typical of trihydroxyandrostanes, which either do not display molecular ions, or if present then they are in minute quantities (Zeitz & Spiteller, 1974). The ions at m/z 290 ($M^+ - 18$) and m/z 272 ($M^+ - 36$) are the result of the loss of the elements of water from $\text{C}_{(12)}$ and $\text{C}_{(3)}$ respectively. The spectrum also displayed a small peak at m/z 275 (8%), which is due to the loss of the hydroxy group at $\text{C}_{(12)}$ and the methyl group at $\text{C}_{(13)}$. Loss of the methyl group at $\text{C}_{(13)}$ leaves the hydroxy group at $\text{C}_{(17)}$ intact, and explains the lack of a significant ion at m/z 254, which would be expected if the hydroxy group at $\text{C}_{(17)}$ were eliminated in the mass spectrometer.

Although mass spectra were not obtained for compounds 14–16, their i.r. spectra all showed a broad peak in the hydroxy-group region. The position and stereochemistry of the hydroxy groups in compounds 14–17 could not be confirmed

conclusively because sufficient material was not available for p.m.r. analysis.

However, since the reduction products of compounds 8 and 10 were identical with compound 14, it was concluded that compound 14 possessed β -hydroxy groups at $\text{C}_{(3)}$, $\text{C}_{(12)}$ and $\text{C}_{(17)}$. Since the reduction products of compounds 9 and 11 were identical with compound 15, it was concluded that compound 15 possessed β -hydroxy groups at $\text{C}_{(3)}$ and $\text{C}_{(17)}$ with an α -hydroxy group at $\text{C}_{(12)}$. Since the reduction product of compound 12 was identical with compound 16, it was concluded that compound 16 possessed β -hydroxy groups at $\text{C}_{(12)}$ and $\text{C}_{(17)}$ with an α -hydroxy group at $\text{C}_{(13)}$, and, since the reduction product of compound 13 was identical with compound 17, it was concluded that compound 17 possessed α -hydroxy groups at $\text{C}_{(3)}$ and $\text{C}_{(12)}$ with a β -hydroxy group at $\text{C}_{(17)}$. The above conclusions are also based on the fact that KBH_4 reduces unconjugated ketone groups to β -orientated hydroxy groups. Compounds 14–17 have therefore been tentatively assigned the structures 5 β -androstan-3 β ,12 β ,17 β -triol, 5 β -androstan-3 β ,12 α ,17 β -triol, 5 β -androstan-3 α ,12 β ,17 β -triol and 5 β -androstan-3 α ,12 α ,17 β -triol respectively.

The final neutral product, compound 18, was recognized as a phenolic compound initially by its u.v. spectrum (λ_{max} , 283 nm), which underwent a bathochromic shift (λ_{max} , 292 nm) on addition of base. The compound, when sprayed with Folin–Ciocalteu reagent on t.l.c. and developed in an atmosphere of ammonia, gave a characteristic blue colour, which is specific for phenolic hydroxy groups (Bush, 1954).

The mass spectrum of compound 18 showed a molecular ion at m/z 300 (M^+ , 39%) and a base peak at m/z 134 (100%), which is typical for 9,10-secosteroids (Jankov, 1977). In addition, the trimethylsilyl ether derivative showed a molecular ion at m/z 372, indicating that the compound possessed one hydroxy group. In this instance the base peak of the spectrum was at m/z 206 (100%), which indicated that the hydroxy group was attached to the A ring. Compound 18 could not be oxidized, but could be reduced and acetylated. This suggested the presence of a hydroxy group and unconjugated ketone groups. This was confirmed by the i.r. spectrum, which displayed two peaks in the unconjugated carbonyl-group region at 1740 and 1705 cm^{-1} and one peak in the hydroxy-group region. The i.r. spectrum also displayed the following peaks at 1608, 1585 and 1500 cm^{-1} (aromatic ring), at 1280 and 1250 cm^{-1} (phenol) and at 855 and 800 cm^{-1} (*o,m*-substituted phenol ring), which are typical of phenolic steroids.

Because compound 18 had M_r 300 and had many of the properties of a 9,10-secosteroid (Dodson & Muir, 1961), it was apparent that, since the initial

substrate possessed a hydroxy group at $C_{(12)}$, either the hydroxy group had been removed or else bond rupture at $C_{(9)}-C_{(10)}$ had occurred with the introduction of a hydroxy group at $C_{(9)}$. It is postulated that 9 α -hydroxy-1,4-dienone steroids are converted into 9,10-secosteroids by a reverse aldol-type reaction (Dodson & Muir, 1961); thus it is unlikely that bond rupture could have occurred without a 9 α -hydroxylation step. It was concluded that the group at $C_{(12)}$ had been removed, and this was augmented by the fact that trimethylsilylation of the compound indicated the presence of only one hydroxy group and indicated that it was attached to the A ring. Finally, compound 18 had m.p. 122°C, similar to that of the secophenol described by Dodson & Muir (1961). Compound 18 has therefore been assigned the structure 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione.

Three major and one minor acidic metabolites were isolated, and structural analyses were performed on the methyl esters.

Compound 19 showed a molecular ion at m/z 400 and a low-intensity ion at m/z 136, suggesting a 4,6-dienone A/B-ring structure (Zaretskii, 1976). Confirmation of this structure was provided by the i.r., u.v. and p.m.r. spectra. Compound 19 was easily oxidized and acetylated, but could not be reduced, suggesting the presence of a hydroxy group. This was confirmed by the i.r. spectrum. The p.m.r. spectrum of the methyl ester of compound 19 showed a triplet centred at δ 5.08 p.p.m., typical of a 12 β -proton. The hydroxy group was therefore assigned the 12 α -configuration. Confirmation for this assignment was provided by the presence of an intense ion (relative to the molecular ion) at m/z 282 in the mass spectrum. An intense ion at m/z 267 (100%) in the mass spectrum of the methyl ester of compound 19 corresponded to the loss of the full bile acid side chain (6C) plus the elements of water. Compound 19 was therefore assigned the structure of 12 α -hydroxy-3-oxochola-4,6-dien-24-oic acid.

The mass spectrum of the methyl ester of compound 20 showed a molecular ion at m/z 398 and a low-intensity ion at m/z 136, indicative of a 4,6-dienone steroid. Confirmation of this structure was provided by the i.r., u.v. and p.m.r. spectra. Compound 20 could not be oxidized or acetylated, but was easily reduced, suggesting the presence of an unconjugated ketone group and the absence of hydroxy groups. This was confirmed by the i.r. spectrum, which displayed two peaks in the carbonyl-group region (indicating the presence of a free carbonyl group (1705 cm^{-1}) in addition to a carboxylic acid carbonyl group (1720 cm^{-1}), but did not display any peaks in the hydroxy-group region. Further confirmation came from the p.m.r. spectrum, which did not show any hydroxy-group protons.

The mass spectrum of the methyl ester of compound 20 also displayed a low-intensity ion at m/z 283 (32%) due to the loss of the full bile acid side chain (6C). Compound 20 is therefore assigned the structure 3,12-dioxochola-4,6-dien-24-oic acid.

The mass spectrum of the trimethylsilyl ether of the methyl ester of compound 21 showed a molecular ion at m/z 444 (M^+ , 10%), but did not display any peaks characteristic of unsaturated steroids. Conversely, the i.r. spectrum (1666, 1618 and 1586 cm^{-1}) and the u.v. spectrum (λ_{max} , 284 nm) of the methyl ester of compound 21 suggested a steroidal 4,6-dienone structure. It is concluded that trimethylsilylation of 4,6-dienone steroids changes the splitting pattern in the mass spectrometer, because this also occurred with the trimethylsilyl derivative of the methyl ester of compound 19. The mass spectrum of the trimethylsilyl ether of the methyl ester of compound 21 also revealed a low-intensity ion at m/z 354 ($M^+ - 90$, 5%) due to the loss of the trimethylsilyl ether function attached to a hydroxy group, and a low-intensity ion at m/z 267 (21%) indicating the loss of a 4C side chain plus trimethylsilyl ether. The actual M_r of this compound was therefore 372, indicating a C_{22} 4,6-dienone steroid with one hydroxy group. The methyl ester of compound 21 could be oxidized and acetylated, but could not be reduced, confirming the presence of a hydroxy group. Because $M^+ - 90$ was of greater intensity than the molecular ion in the mass spectrum of the trimethylsilyl ether of the methyl ester of compound 21, it is suggested that the hydroxy group at $C_{(12)}$ was in the α -configuration. Compound 21 has therefore been assigned the structure 12 α -hydroxy-3-oxopregna-4,16-diene-20-carboxylic acid.

The methyl ester of compound 22 was found to be identical with methyl 7 α ,12 α -dihydroxy-3-oxo-5 β -cholan-24-oate synthesized by the method of Tserng (1978) in its t.l.c. and g.l.c. behaviour. The methyl ester of compound 22 was easily oxidized, acetylated and reduced, indicating the presence of hydroxy groups and an unconjugated ketone group. This was confirmed by the i.r. spectrum. The mass spectrum revealed a low-intensity molecular ion at m/z 420 (M^+ , 13%), low-intensity ions at m/z 402 (42%) and m/z 384 (20%) and intense ions at m/z 287 ($M^+ - 115 + \text{H}_2\text{O}$, 100%), indicating the loss of the full bile acid side chain (6C). The p.m.r. spectrum did not reveal any vinylic protons; however, a triplet was present at δ 4.00 p.p.m., typical of a 12 β -proton on a carbon carrying a 12 α -hydroxy group, and a broad singlet at δ 4.07 p.p.m., typical of a 7 β -proton on a carbon carrying a 7 α -hydroxy group. The mass and p.m.r. spectra indicate therefore that the hydroxy groups in compound 22 were in the 7 α - and 13 α -positions. Compound 22 has thus been assigned

the structure 7 α ,13 α -dihydroxy-3-oxo-5 β -cholan-24-oic acid.

Discussion

Although many unsaturated bacterial metabolites of cholic acid have been described (Severina *et al.*, 1968, 1969; Tenneson *et al.*, 1979c), the present paper is only the second report of C₁₉ androstanes with a 4,6-dienone structure. Metabolites 3–5 are therefore novel compounds. Additionally, this study reveals that *Pseudomonas* sp. N.C.I.B. 10590, although an obligate aerobe, has the ability to degrade cholic acid under anaerobic conditions. Aerobic metabolism of bile acids by this organism is well documented (Tenneson *et al.*, 1978a,b, 1979a,b,c). The organism can in fact grow to stationary phase with bile acids as sole carbon source under aerobic conditions and can fully metabolize bile acids (0.1%) in 24 h (chenodeoxycholic acid) to 32 h (hyodeoxycholic acid). The organism will not grow and proliferate on bile acids under anaerobic conditions; however, resting-cell suspensions are known to degrade chenodeoxycholic acid (Owen *et al.*, 1983a) and hyodeoxycholic acid (Owen & Bilton, 1983) under these conditions.

Metabolism of cholic acid (Tenneson *et al.*, 1979c) by *Pseudomonas* sp. N.C.I.B. 10590 under aerobiosis gives predominantly metabolites with a 1,4-dienone structure that still retain the 7 α - and 12 α -hydroxy groups.

The present study reveals that under anaerobic conditions induction of a 7 α -dehydroxylase enzyme occurs, so that the predominant metabolites have a 4,6-dienone structure. Thus cholic acid, like chenodeoxycholic acid (Owen *et al.*, 1983a), appears to enhance the induction of a 7 α -dehydroxylase enzyme.

When the fermentation was terminated after 6 weeks' incubation, nitrate and nitrite ions could not be detected in the medium, indicating that the electron-acceptor supply had been exhausted. Time-course studies revealed that this occurred in the fifth week of fermentation and coincided with the appearance of saturated androstanes in the medium, which probably arise by hydrogenation of 1,4,6-trienone and 4,6-dienone steroids via 1,4-dienone and 4-en-3-one steroids. The isolation of 12 α -hydroxyandrosta-1,4-diene-3,17-dione from the culture indicates that this is the case. It is probable that hydrogenation occurs as a means of regenerating oxidative power by the concomitant oxidation of NADH to NAD⁺ (cofactors that are known to be required for hydroxy steroid dehydrogenation). Although this organism cannot grow under anaerobic conditions, nevertheless it remains viable and

can be subcultured on to aerobic plates after several months of anaerobic culture (R. F. Bilton, unpublished work).

The anaerobic transformation of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590 appears to follow a similar pattern to that for other bile acids in that the pattern of enzymic events involves hydroxy steroid dehydrogenation at C₍₃₎, nuclear steroid dehydrogenation followed by side-chain cleavage from unsaturated C₂₄ acidic via C₂₂ acidic to C₁₉ neutral androstanes. Epimerization of the 12 α -hydroxy group to a 12 β -hydroxy group appears to be essential for side-chain cleavage to occur (Tenneson *et al.*, 1979c). It appears that the anaerobic pathway involves the induction of a further enzyme, namely 7 α -dehydroxylase, thus giving 4,6-dienones as the major metabolites. This enzyme requires at least a 7 α -hydroxy-4-en-3-one substrate, because compounds possessing a double bond at C₍₆₎–C₍₇₎ only were not isolated. The pathway appears to be reversed somewhat when KNO₃ runs out, thus bringing about the reversal of nuclear steroid dehydrogenation, thereby giving rise to saturated androstanes.

Isolation of a secosteroid (compound 18) indicates that cleavage of the steroid nucleus can also occur under anaerobic conditions. An identical compound has been isolated after the bacterial degradation of androstanes under aerobic conditions (Dodson & Muir, 1961; Miclo & Germain, 1978). These workers showed that the production of the secosteroid occurred via 9 α -hydroxy intermediates.

It is probable that compound 18 was produced from the unsaturated androstanes present in the medium. However, all the androstanes possessed a hydroxy group at C₍₁₂₎. To produce compound 18, the hydroxy group at C₍₁₂₎ must have been removed. Studies with the intermediate 12 β -hydroxyandrosta-1,4-diene-3,17-dione showed that the pseudomonad also removed the hydroxy group at C₍₁₂₎ during the production of secosteroids under aerobic conditions. The secosteroid isolated in the aerobic studies was assigned the structure 3-hydroxyandrosta-1,3,5(10),11-tetraene-9,17-dione (compound 23). The double bond at C₍₁₁₎–C₍₁₂₎ indicated that removal of the hydroxy group was mediated by 12 α -dehydroxylation. The double bond at C₍₁₁₎–C₍₁₂₎ is probably reduced under anaerobic conditions, thus giving rise to compound 18. Therefore it appears that hydroxy groups at C₍₁₂₎ inhibit 9 α -hydroxylation in some way and must be removed for ring fission of such compounds to take place. This discovery thus rules out the possibility that C₍₁₂₎-hydroxy groups assist 9 α -hydroxylation under anaerobic conditions by a process of trans-hydroxylation. However, the double bond at C₍₁₁₎–C₍₁₂₎ may assist such a process by a previously

proposed mechanism (Owen *et al.*, 1983b) that would not require molecular O₂.

In conclusion, *Pseudomonas* sp. N.C.I.B. 10590 degrades cholic acid under anaerobic conditions, producing C₂₄, C₂₂ and C₁₉ metabolites. The major unsaturated metabolites possess a 4,6-dienone structure that can be hydrogenated in the absence of a terminal electron receptor. The unsaturated androstanes can also be aromatized to secosteroids, which are probably metabolized to non-steroidal products (Miclo & Germain, 1978).

The pseudomonad thus provides a useful model system for studying the mechanism of bile acid degradation under anaerobic conditions, and the analytical data on the various metabolites described in this paper may be useful in aiding their detection in faeces and colonic biopsies from colon-cancer patients. This may enable the hypotheses of Aries *et al.* (1969) and Hill *et al.* (1978) to be investigated more fully.

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