Biotransformation of ursodeoxycholic acid by *Pseudomonas* sp NCIB 10590

Robert W. Owen,[1,*] Robin Wait,[*] and Rodney F. Bilton[†]

Department of Chemistry and Biochemistry,[†] Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, England, and PHLS, Centre for Applied Microbiology and Research,[*] Bacterial Metabolism Research Laboratory, Porton Down, Salisbury, Wiltshire SP4 0JG, England

Abstract The metabolism of ursodeoxycholic acid by *Pseudomonas* sp NCIB 10590 has been studied in phosphate-buffered mineral salts. The organism completely metabolized ursodeoxycholic acid in 24 hr, and time-course experiments revealed that maximum product formation occurred at 14 hr. The major products detected and identified at 14 hr were 7β-hydroxychol-4-en-3-one-24-oic acid, 7β-hydroxy-3-oxo-pregna-1,4-diene-20-carboxylic acid, and 7β-hydroxyandrosta-1,4-diene-3,17-dione. Several minor intermediates were isolated and evidence is given for the following structures: 7β-hydroxy-5β-cholan-3-oxo-24-oic acid, 7β-hydroxyandrost-4-en-3,17-dione, 7β,17β-dihydroxyandrosta-1,4-diene-3-one, 3-hydroxy-1,3,5(10)-9,10-seco-androstatriene-3,17-dione-7β-ol, and 22α-hydroxymethylpregna-1,4-diene-3-one-7β-ol. **—Owen, R. W., R. Wait, and R. F. Bilton.** Biotransformation of ursodeoxycholic acid by *Pseudomonas* sp NCIB 10590. *J. Lipid Res.* 1988. **29:** 459–468.

Supplementary key words bile acids • nuclear magnetic resonance • mass spectrometry • infrared spectrophotometry

Ursodeoxycholic acid (UDCA) is a major biliary bile acid of some species of bears (1) and has also been detected in human bile (2) and feces (3). The pharmacology of UDCA has been described in detail (4, 5) because it is now used as a drug for the dissolution of radiolucent gallstones in man. When UDCA is orally administered to man, the bile becomes enriched with UDCA so that it may represent up to 60% of biliary bile acids (5). The mechanism of action of UDCA may rely, in part, on being transformed to chenodeoxycholic acid (CDCA) which is also used for the dissolution of gallstones (6–8). However, due to side effects of CDCA treatment, UDCA is now the drug of choice. UDCA may be converted to CDCA during the enterohepatic circulation; that which is not absorbed in the terminal ileum enters the colon where it is subjected to two types of bacterial enzymic attack. UDCA can be oxidized by 7β-hydroxysteroid dehydrogenase (7β-HSDH) to give 3α-hydroxy-5β-7-oxo-cholanoic acid (7KLA) which can be reduced to CDCA either by the colonic flora (9) or by cytochrome P450-mediated hepatic enzymes (10) after passive absorption from the colon. UDCA can also be dehydroxylated by colonic bacteria to lithocholic acid which is subsequently excreted in feces (11).

The use of UDCA as a chemotherapeutic drug has led many research groups to study the metabolism of this bile acid by bacteria. Much of the work so far has concentrated on the metabolism of UDCA by bacteria indigenous to the human intestinal tract.

Macdonald et al. (12) have shown that UDCA can be converted to CDCA via 7KLA by *Clostridium absonum;* this epimerization reaction is substantiated by several other studies (13, 14). UDCA is also known to be epimerized at C_3; this is demonstrated in twenty species of *Clostridium perfringens* by Hirano et al. (15).

To date there is no evidence for extensive metabolism of UDCA by bacteria. Hence a study has been conducted on the biotransformation of UDCA by *Pseudomonas* sp NCIB 10590 (*Pseudomonas* sp 10590).

MATERIALS

5α-Cholestane and androsta-1,4-diene-3,17-dione were obtained from Koch Light Laboratories (Colnbrook, Bucks, England) while UDCA was obtained from Sigma Chemical Co. (Poole, Dorset, England). General reagents obtained from M W Scientific, Eastleigh, Hants, England were of Analar grade and solvents were redistilled before use.

Abbreviations: *Pseudomonas* sp 10590, *Pseudomonas* sp NCIB 10590; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; MS, mass spectrum; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; ADD, androsta-1,4-diene-3,17-dione; TMS, trimethylsilyl.

[1]To whom reprint requests should be addressed.

METHODS

Melting points (mp) were determined using a Buchi 510 melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were determined for solutions in methanol on a Pye-Unicam SP 1800 spectrophotometer. Infrared (IR) spectra were determined from KBr discs on a Unicam SP 1200 spectrophotometer. Nuclear magnetic resonance (NMR) proton spectra were recorded on a Jeol PMX 60SI spectrometer operating at 60 mHz at 30°C from solutions in deuterated chloroform. Mass spectra (MS) were obtained using a Dupont 21-491 series mass spectrometer either by direct inlet, or by combined GLC–mass spectrometry using a Varian Aerograph 2700 gas chromatograph. Data reduction was performed on a Hewlett-Packard dual disc system. Gas–liquid chromatography (GLC) and thin-layer chromatography (TLC) were conducted as described previously (16).

Biotransformation medium

The culture medium contained (g/l of distilled water) sodium ursodeoxycholate, 1.0; K_2HPO_4, 0.7; KH_2PO_4, 0.3; KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.0025; $ZnSO_4 \cdot 7H_2O$, 0.0025; and $MnSO_4 \cdot 4H_2O$, 0.0025; final pH, 7.4. Solutions of sodium ursodeoxycholate, mineral salts, trace elements, and magnesium sulfate were autoclaved separately at 120°C for 15 min prior to admixture. The medium was dispensed as 1-liter aliquots into 2-liter Erlenmeyer shake flasks prior to inoculation.

Fermentation

The medium was spiked with a 10% inoculum of a stationary phase culture of *Pseudomonas* sp 10590 that had been grown on 0.1% sodium ursodeoxycholate for 24 hr. Fermentation was conducted on an LH Engineering orbital incubator, shaken at 200 rev/min for 24 hr at 28°C. The course of the biotransformation was followed by sampling duplicate cultures at 1-hr intervals. Five-ml aliquots were pooled and the cell density was calculated from the absorbance of the culture at 540 nm. Further 5-ml aliquots were pooled for filtration of the sample through a 0.2-μm Millipore filter, which enabled the direct determination of the absorbance at 252 nm, indicating the amount of unsaturated steroid present in the culture. Finally 5-ml aliquots were pooled, acidified to pH 4.0, and extracted twice with ethyl acetate for TLC and GLC analysis. GLC analysis enabled the quantitation of ursodeoxycholic acid and the major acidic and neutral intermediates that were produced. When the concentration of unsaturated intermediates reached a plateau at 14 hr, 300-ml aliquots were removed from duplicate flasks, acidified to pH 4.0, and extracted twice with ethyl acetate (600 ml) for product analysis. After drying over anhydrous $MgSO_4$, the solvent was removed under reduced pressure at 50°C to yield 260 mg of a tarry residue. The residue was dissolved in warm 72% aqueous ethanol, passed through Amberlyst A-15 to remove cations, and fractionated into neutral and acidic steroids by DEAP-LH-20 column chromatography (17). The solvent was removed from the neutral (75 mg) and acidic (165 mg) fractions by rotary evaporation, which were subsequently dried to constant weight in vacuo at 37°C. The acidic fraction was separated by preparative TLC (15 plates) in the solvent system 2,2,4-trimethylpentane–ethyl acetate–glacial acetic acid 4.5:4.5:1 (v/v) yielding residual UDCA (28 mg) and a series of fractions from which steroids 2–4 were crystallized. The neutral fraction was separated by preparative TLC (seven plates) in the solvent system methanol–dichloromethane 1:19 (v/v) yielding a series of fractions from which steroids 5–9 were crystallized.

Steroid derivatization

Acidic steroids were methylated using ethereal diazomethane prior to GLC, NMR, and MS and, when appropriate, steroids were silylated using trimethylsilyl imidazole. Oximes were prepared by addition of 5 μl of methoxyamine–HCl (20 mg/ml solution in pyridine) to the dried sample and allowing to stand at room temperature overnight (18). The samples were dried under a stream of nitrogen and silylated as above.

Methyl 7β-hydroxy-5β-cholan-3-oxo-24-carboxylate (methyl ester of metabolite 2). Recrystallization of the methyl ester of metabolite 2 from methanol–dichloromethane (see Fig. 3) gave an oily residue (10 mg); IR 3360 (hydroxyl), 1730 (carboxyl), and 1710 cm^{-1} (3-ketone); NMR, (δ) 0.70, 1.20 (6H, s, 18-CH_3 and 19-CH_3), 0.94 (3H, s, 21-CH_3), 3.40 (1H, m, 7-H), and 3.54 (3H, s, 24-OCH_3); MS, M^+ 404 (7%, $C_{25}H_{40}O_4$ requires M^+ 404), base peak at m/e 271 (M^+-133, 6C-side chain + H_2O, 100%); GLC, RRT 12.09; TLC, R_f 0.51; after oxidation, R_f 0.73; after acetylation, R_f 1.10; and after reduction, R_f 0.14.

Methyl 7β-hydroxy-3-oxochol-4-ene-24-carboxylate (methyl ester of 3). Recrystallization of the methyl ester of metabolite 3 from methanol–dichloromethane (see Fig. 3) gave white powdery crystals (40 mg) mp 198°C (free acid); IR 3455 (hydroxyl), 1710 (carboxyl), 1645 (3-ketone), and 1605 cm^{-1} (C_4–C_5 double bond); UV, 242 nm (ξ16,000); NMR, (δ), 0.70, 1.27 (6H, s, 18-CH_3 and 19-CH_3), 0.92 (3H, d, J=5Hz, 21-CH_3) 3.40 (1H, m, 7-H), 3.52 (3H, s, 24-OCH_3) and 5.58 (1H, s, 4-H); MS, M^+ 402 (19%, $C_{25}H_{38}O_4$ requires M^+ 402), base peak at m/e 384 (M^+–18, H_2O, 100%) and intense ions at m/e 269 (M^+–133, 6C-side chain + H_2O, 55%) and 124 (4-ene-3-one, 98%): MS (O-methyl-oxime-TMS), M^+ 503 (66%, $C_{25}H_{38}O_4$ + TMS + O-methyl oxime requires M^+ 503), low intensity ion at m/e 472 (M^+–31, O-methyl oxime, 13%), and base peak at m/e 382 (M^+–121, TMS + O-methyl oxime, 100%); GLC RRT 11.37; TLC, R_f 0.46; after oxidation, R_f 0.69; after acetylation, R_f 1.06; and after reduction, R_f 0.46.

Methyl 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylate (methyl ester of 4). Recrystallization of the methyl ester of metabolite 4 from methanol–dichlormethane (see Fig. 3) gave white crystals (41 mg) mp 260°C (free acid); IR, 3,400 (hydroxyl), 1700 (carboxyl), 1660 (3-ketone), 1608 and 1600 cm^{-1} (C_1–C_2 and C_4–C_5 double bonds) UV, 244 nm (ξ 15,000); NMR (δ), 0.76, 1.24 (6H, s, 18–CH_3 and 19–CH_3), 1.08 (3H, d, J=5Hz, 21–CH_3), 3.32 (1H, m, 7–H), 3.56 (3H, s, 22–OCH_3), 5.96 (1H, s, 4–H), 6.06 (1H, d, showing further splitting, J=10Hz, 2–H), and 6.80 (1H, d, j=10Hz, 1–H); MS, M^+ 372 (15%, $C_{23}H_{32}O_4$ requires M^+ 372), base peak at m/e 122 (1,4-diene-3-one, 100%) and low intensity ions at m/e 354 (M^+–18, H_2O, 7%) and at m/e 267 (M^+–105, 4c-side chain + H_2O, 4%): MS (TMS–ether) M^+ 444 (5%, $C_{23}H_{32}O_4$ + TMS requires M^+ 444), low intensity ion at m/e 354 (M^+–90, TMS, 18%), base peak at m/e 323 (M^+–121, A-ring fragment, 100%) and low intensity ion at m/e 267 (M^+–4C-side chain + TMS, 15%); MS (O-methyl oxime-TMS), M^+ 473 (38%, $C_{23}H_{32}O_4$ + 2 O-methyl oxime + TMS requires M^+ 473), low intensity ions at m/e 458 (M^+–15, CH_3, 8%), m/e 442 (M^+–31, OCH_3, 8%), m/e 383 (M^+–90, TMS, 12%) and base peak at m/e 352 (M^+–121, OCH_3 + TMS, 100%); GLC, RRT 6.31; TLC, R_f 0.41; after oxidation, R_f 0.62; after acetylation, R_f 1.02; and after reduction, R_f 0.41.

7β-Hydroxyandrosta-1,4-diene-3,17-dione (6). Recrystallization of metabolite 6 (see Fig. 4) from methanol–dichloromethane gave white spikey needles (28 mg); mp 200°C; IR, 3360 (hydroxyl), 1740 (17-ketone), 1660 (3-ketone), 1608 and 1600 cm^{-1} (C_1–C_2 and C_4–C_5 double bonds); UV, 244 nm (ξ15,000); NMR (δ), 0.92, 1.25 (6H, s, 18–CH_3 and 19–CH_3), 2.45–2.51 (2H, m, 16–CH_2), 3.45 (1H, m, 7–H), 5.91 (1H, s, 4–H), 5.95 (1H, d, showing further splitting, J=10Hz, 2–H), 6.79 (1H, d, J=10Hz, 1–H); MS, M^+ 300 (20%, $C_{19}H_{24}O_3$ requires M^+ 300), base peak at m/e 122 (1,4-diene-3-one, 100%) and low intensity ion at m/e 282 (M^+–18, H_2O, 12%); MS (O-methyl oxime-TMS), M^+ 430 (66%, $C_{19}H_{24}O_3$+ 2 O-methyl oxime + TMS requires M^+ 430), low intensity ions at m/e 415 (M^+–15, CH_3, 20%), m/e 399 (M^+–31, OCH_3, 18%) m/e 340 (M^+–90, TMS, 20%) and intense ion at m/e 309 (M^+–121, OCH_3 + TMS, 90%); GLC, RRT 2.71; TLC, R_f 0.59; after oxidation, R_f 1.01; after acetylation, R_f 1.05; and after reduction, R_f 0.38.

7β-Hydroxyandrost-4-ene-3,17-dione (5). Recrystallization of metabolite 5 (see Fig. 4) from methanol–dichloromethane gave white needles (10 mg); mp 216–218°C, IR, 3349 (hydroxyl), 1736 (17-ketone), 1642 (3-ketone) and 1612 cm^{-1} (C_4–C_5 double bond); UV, 242 nm (ξ 16,000); NMR (δ), 0.92, 1.20 (6H, s, 18–CH_3 and 19–CH_3), 2.46–2.56 (2H, m, 16–CH_2), 3.50 (1H, m, 7H), 5.58 (1H, s, 4–H); MS, M^+ 302 (base peak, 100%, $C_{19}H_{26}O_3$ requires M^+ 302), intense ion at m/e 124 (4-ene-3-one, 72%) and low intensity ion at m/e 284 (M^+–18, H_2O, 20%); MS (O-methyl oxime-TMS), base peak molecular ion at m/e 432 (100%, $C_{19}H_{26}O_3$ + 2 O-methyl oxime + TMS requires M^+ 432), low intensity ions at m/e 417 (M^+–15, CH_3, 39%), and m/e 401 (M^+–31, OCH_3, 48%), and intense ions at m/e 342 (M^+–90, TMS, 55%); m/e 327 (M^+–105, TMS + CH_3, 58%) and at m/e 311 (M^+–121, OCH_3+TMS, 99%); GLC, RRT 2.49; TLC, R_f 0.68; after oxidation, R_f 1.03; after acetylation, R_f 1.07; and after reduction, R_f 0.45.

7β,17β-Dihydroxyandrosta-1,4-diene-3-one (7). Recrystallization of metabolite 7 (see Fig. 4) from methanol–dichloromethane gave white crystals (3 mg); IR, 3350, 3330 (hydroxyls), 1739 (3-ketone), 1610 and 1600 cm^{-1} (C_1–C_2 and C_4–C_5 double bonds); UV, 244 nm (ξ15,000); NMR (δ), 0.82, 1.24 (6H, s, 18–CH_3 and 19–CH_3), 2.50–2.58 (2H, m, 16–CH_2), 3.42 (1H, t, J=8H, 17–H), 3.62 (1H, m, 7H), 5.94 (1H, s, 4–H) 6.14 (1H, d, showing further splitting, J=10Hz, 2–H), 6.78 (1H, d, J=10Hz, 1–H); MS (TMS–ether), M^+ 446 (11%, $C_{19}H_{26}O_3$ + 2 TMS requires M^+ 446), base peak at m/e 325 (M^+–121, A-ring fragment, 100%) and low intensity ions at m/e 356 (M^+–90, TMS, 33%) and m/e 266 (M^+–180, 2 TMS, 23%): MS (O-Me-oxime-TMS), M^+ 475 (81%, $C_{19}H_{26}O_3$ + O-methyl oxime + 2 TMS requires M^+ 475), low intensity ions at m/e 460 (M^+–15, CH_3, 43%) m/e 444 (M^+–31, OCH_3, 41%), m/e 385 (M^+–90, TMS, 45%), base peak at m/e 354 (M^+–121, OCH_3 + TMS, 100%) and low intensity ion at m/e 295 (M^+–180, 2 TMS, 40%); GLC, RRT 2.78; TLC, R_f 0.38; after oxidation, R_f 1.01; after acetylation, R_f 1.31; and after reduction, R_f 0.38.

3-Hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione-7β-ol (8). Recrystallization of metabolite 8 (see Fig. 4) from methanol–dichloromethane gave an oily residue (5 mg); IR, 3465 (hydroxyl), 1730 (17-ketone), 1700 (9-ketone), 1615, 1585, 1500 (aromatic ring), 1285, 1255 (phenol) 850 and 810 cm^{-1} (*a,m*-substituted phenol ring); UV, 220 nm, 283 nm, UV NaOH/MeOH 240 nm, 296 nm; NMR (δ), 1.22, 1.88 (6H, s, 18–CH_3 and 19–CH_3), 4.10 (1H, m, 7–H), 4.32 (1H, s, 3H), 6.48 (1H, s,4–H), 6.60 (1H, q, 2–H), 6.90 (1H, d, J=8Hz, 1–H); MS (TMS–ether), M^+ 460, 13% ($C_{19}H_{24}O_3$ + 2TMS requires M^+ 460), low intensity ion at m/e 445 (M^+–15, CH_3 3%), low intensity ion at m/e 370 (M^+–90, TMS, 20%) and base peak at m/e 267 (M^+–193, A-ring fragment, 100%): MS (O-Me-oxime), M^+ 518, 55% ($C_{19}H_{24}O_3$ + 2TMS + 2 O-Me-oxime requires M^+518), intense ion at m/e 503 (M^+–15, CH_3, 53%), base peak at m/e 428 (M^+–90, TMS, 100%), and intense ions at m/e 397 (M^+–121, TMS + O-Me-oxime, 76%) and low intensity ion at m/e 295 (M^+–223, C+D rings, 86%); GLC, RRT 1.75; TLC, R_f 0.73; after oxidation, R_f 1.33; after acetylation, R_f 1.37; and after reduction, R_f 0.40.

22-Hydroxymethylpregna-1,4-diene-3-one-7β-ol (9). Recrystallization of metabolite 9 (see Fig. 4) from methanol–dichloromethane gave white crystals (3 mg); IR, 3440

(hydroxyl), 1660 (3-ketone), 1610 and 1600 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); UV, 245 (ξ 16,000); NMR (δ), 0.74, 1.22 (6H, s, 18-CH_3 and 19-CH_3), 1.05 (3H, s, 21-CH_3), 3.50 (1H, m, 7-H), 6.07 (1H, s, 4-H), 6.20 (H, d, showing further splitting, J=10Hz, 2-H), 7.06 (1H, d, J=10Hz, 1-H); MS, M^+ 344 (36%, $C_{22}H_{32}O_3$ requires M^+ 344), base peak at m/e 122 (1,4-diene-3-one, 100%) and low intensity ion at m/e 326 (M^+-18, H_{20}, 11%): MS (TMS-ether), M^+ 488 (11% $C_{22}H_{32}O_3$ + 2TMS requires M^+ 488), low intensity ion at m/e 398 (M^+-90, TMS, 11%), base peak at m/e 367 (M^+-121, A-ring fragment, 100%), low intensity ion at m/e 308 (M^+-180, 2TMS, 5%) and low intensity ion at m/e 267 (M^+-221, 3C-side chain + 2TMS, 33%): MS (O-Me-oxime), base peak molecular ion at m/e 517 (100%, $C_{22}H_{32}O_3$ + 2TMS + O-methyl oxime requires M^+ 517), intense ions at m/e 486 (M^+-31, O-methyl oxime, 66%), at m/e 427 (M^+-90, TMS, 67%), at m/e 396 (M^+-121, TMS + O-methyl oxime, 94%), and at m/e 306 (M^+-211, 2TMS + O-methyl oxime, 90%); GLC, RRT 2.82; TLC, R_f 0.43; after oxidation, R_f 0.63; after acetylation, R_f 1.35; and after reduction, R_f 0.43.

RESULTS

Pseudomonas sp 10590 grew rapidly on sodium ursodeoxycholate in a mineral salts medium. The course of the biotransformation was followed by measurement of the increase in both cell density and the concentration of 1,4-diene-3-oxo steroids (**Fig. 1**). The growth of *Pseudomonas* sp 10590 on sodium ursodeoxycholate showed typical lag, log, and stationary phases, while the concentration of 1,4-diene-3-oxo steroids in the medium showed a maximum after 15 hr. The log phase of growth was biphasic, with maximum growth of the Pseudomonad occurring as the concentration of 1,4-diene-3-oxo steroids was decreasing in the medium. The concentration of ursodeoxycholate was shown to decrease with time, whereas the concentration of the major acidic and neutral 1,4-diene-3-one steroids reached a maximum after 13 and 14 hr, respectively (**Fig. 2**). Eight metabolites (three acidic and five neutral) were isolated and the assigned structures are listed in **Fig. 3** and **Fig. 4.**

The mass spectrum (**Fig. 5a**) of one of the major acidic metabolites, 4, gave an intense ion (base peak) at m/e 122

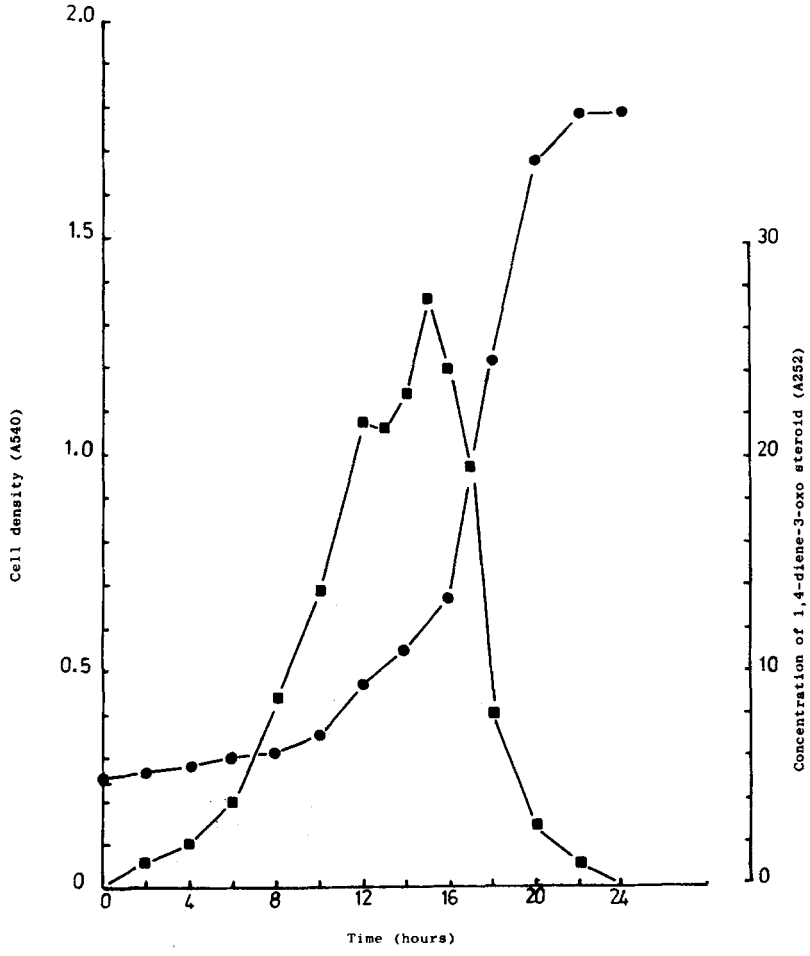

Fig. 1. The relationship between cell density (●) and concentration of 1,4-diene-3-oxo steroid (■) during the oxidation of ursodeoxycholic acid (1) by *Pseudomonas* sp NCIB 10590.

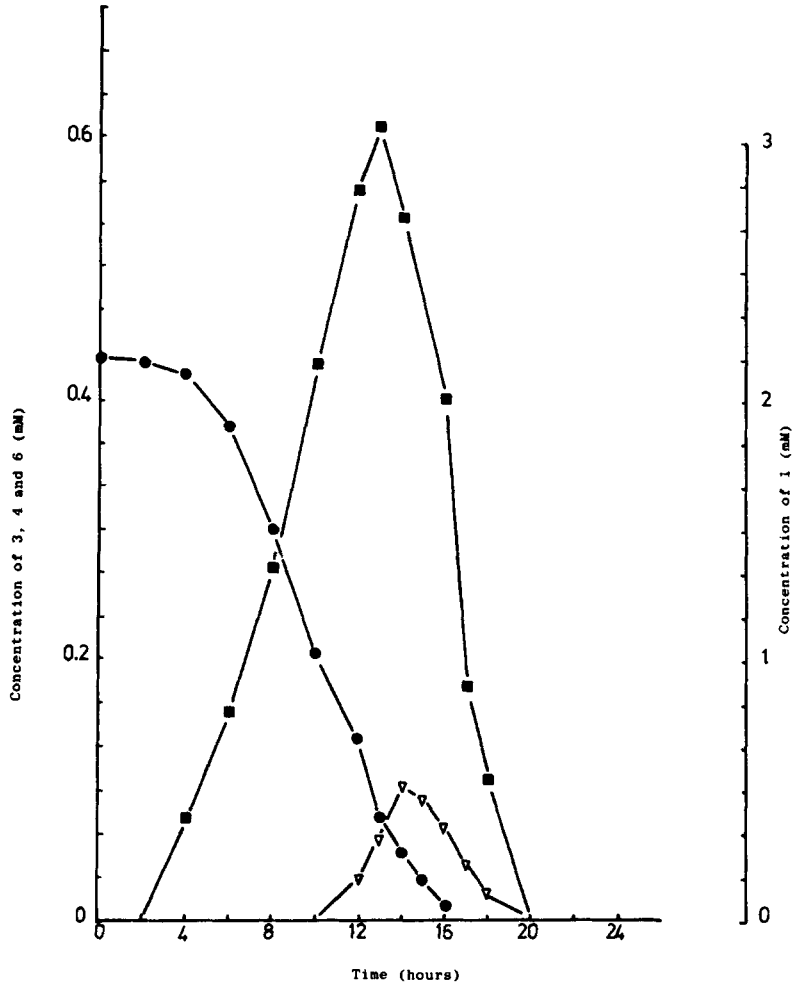

Fig. 2. The relationship between the concentration of ursodeoxycholic acid (1) (●), the major acidic (3 and 4) (■), and neutral (6) (▽) metabolites during the degradation of ursodeoxycholic acid by *Pseudomonas* sp NCIB 10590.

that is typical for a steroidal 1,4-dien-3-one A-ring structure (19). Confirmation of this structure was provided by the UV spectrum (λ max, 244 nm, di-β-substituted αβ-unsaturated ketone in a six-membered ring, double bond exocyclic (20), IR spectrum (1660, 1608, and 1600 cm^{-1}, αβ-unsaturated ketone) and NMR spectrum (three vinylic protons in the range 5.96–6.80 δ). Metabolite 4 was easily oxidized and acetylated but could not be reduced, suggesting the lack of an unconjugated ketone group but the presence of an hydroxy group. This was confirmed by the IR spectrum which displayed a peak at 3400 cm^{-1} characteristic of hydroxyl groups with the absence of any peaks in the unconjugated carbonyl region.

Further confirmation was obtained from *1*) the mass spectrum of the TMS-ether (Fig. 5b) which gave a molecular ion at m/e 444 (5%) consistent with the addition of TMS to a hydroxyl group, and *2*) the mass spectrum (Fig. 5c) of the O-methyl oxime-TMS ether which gave a molecular ion at m/e 473 (38%) consistent with the addition of TMS to a hydroxyl group and O-methyl oxime to a ketone group.

The position and stereochemistry of the hydroxyl group in metabolite 4 was assigned from the NMR spectrum. It has been shown that protons associated with carbon atoms carrying hydroxyl groups in certain steroids give rise to characteristic splitting patterns (21). The NMR spectrum of metabolite 4 displayed a broad (multiplet) peak centered at 3.32 δ which accords with the position and pattern for a 7α-proton attached to C_7 on the steroid nucleus (21). The hydroxyl group in metabolite 4 was thus assigned the 7β-configuration. With a low intensity ion at m/e 267 (4%) in the mass spectrum suggesting the loss of a 4C-side chain, metabolite 4 has been assigned the structure 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylic acid.

The mass spectrum of the second major metabolite, 3, gave an intense ion at m/e 124 that is typical for a steroidal 4-ene-3-one A-ring structure (19). Confirmation of this structure was provided by the UV spectrum (λ max, 242

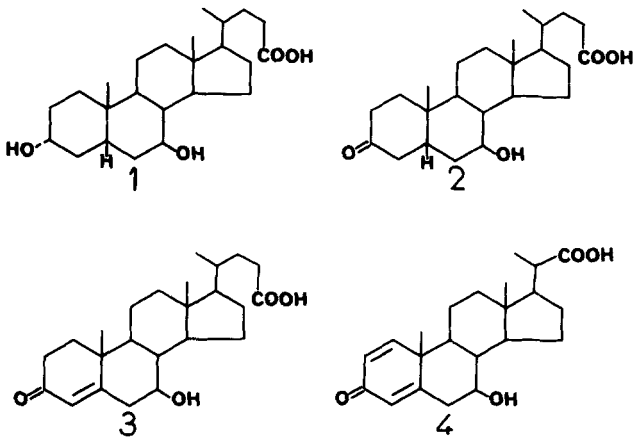

Fig. 3. Acidic metabolites isolated after the aerobic biotransformation of ursodeoxycholic acid (1) by *Pseudomonas* sp NCIB 10590. 1: Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid); 2: 7β-hydroxy-3-oxo-5β-cholan-24-oic acid; 3: 7β-hydroxy-3-oxochol-4-ene-24-oic acid; 4: 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylic acid.

nm, di-β-substituted α,β-unsaturated ketone in a six-membered ring, double bond exocyclic (20), IR spectrum (1645 and 1605 cm^{-1}, αβ-unsaturated ketone) and NMR spectrum (one vinylic proton at 5.58 δ). Reactions on TLC revealed that 3 possessed a hydroxyl group and lacked an unconjugated ketone group. This was confirmed by the IR spectrum, and the mass spectrum of the O-methyl oxime-TMS ether derivative.

The NMR spectrum of metabolite 3 displayed a broad (multiplet) peak centered at 3.40 δ which accords with the position and pattern for a 7α-proton attached to C_7 on the steroid nucleus (21). With an intense ion at m/e 269 (55%) in the mass spectrum suggesting the loss of a 6C-side-chain, metabolite 3 has been assigned the structure 7β-hydroxy-3-oxochol-4-ene-24-oic acid.

The mass spectrum of the minor acidic metabolite 2 revealed a molecular ion at m/e 404 (M^+, 7%) and a base peak at m/e 271 (100%) due to the loss of the full bile acid side chain (6C-side-chain + H_2O). The methyl ester of metabolite 2 was easily oxidized, acetylated, and reduced on TLC indicating the presence of a hydroxyl group and an unconjugated ketone group. This was confirmed by the IR spectrum. Further confirmation was provided by the mass spectrum of the O-methyl oxime-TMS ether derivative which displayed a molecular ion at m/e 505 (M^+, 2%), a base peak at m/e 474 (100%) due to the loss of OCH_3 from a ketone group, and a low intensity ion at m/e 415 (8%) due to the loss of TMS. The NMR spectrum did not reveal vinylic protons; however a broad multiplet was present at 3.40 δ typical of a 7α-proton on a carbon carrying a 7β-hydroxyl group. Metabolite 2 was therefore assigned the structure 7β-hydroxy-3-oxo-5β-cholan-24-oic acid.

The mass spectrum (**Fig. 6a**) of the major neutral metabolite 6 gave an intense ion (base peak) at m/e 122 that is typical for a steroidal 1,4-diene-3-one A-ring structure (19). Confirmation of this A-ring structure was provided by the UV spectrum (λ max, 244 nm, di-β-substituted αβ-unsaturated ketone in a six-membered ring, double bond exocyclic) (20), the IR spectrum (1660, 1608, and 1600 cm^{-1}, αβ-unsaturated ketone) and the NMR spectrum (three vinylic protons in the range 5.91–6.79 δ).

Metabolite 6 was easily oxidized and acetylated and was reduced with potassium borohydride to a product that corresponded in R_{fADD} and color (purple mauve) to metabolite 7 on TLC. This indicated the possession of both a hydroxyl and an unconjugated ketone group. This was confirmed by the IR spectrum which displayed major peaks in both the hydroxyl region (3360 cm^{-1}) and the carbonyl region (1740 cm^{-1}) characteristic of a ketone group in a five-membered ring. Further confirmation was obtained from the mass spectrum (Fig. 6b) of the O-methyl oxime-TMS ether which gave a molecular ion at m/e 430 (M^+, 66%) consistent with the addition of TMS to a hydroxyl group and O-methyl oxime to two distinct ketone groups (3-ketone plus one unconjugated ketone group). The NMR spectrum of metabolite 6 showed a broad multiplet centered at 3.45 δ and on this basis the

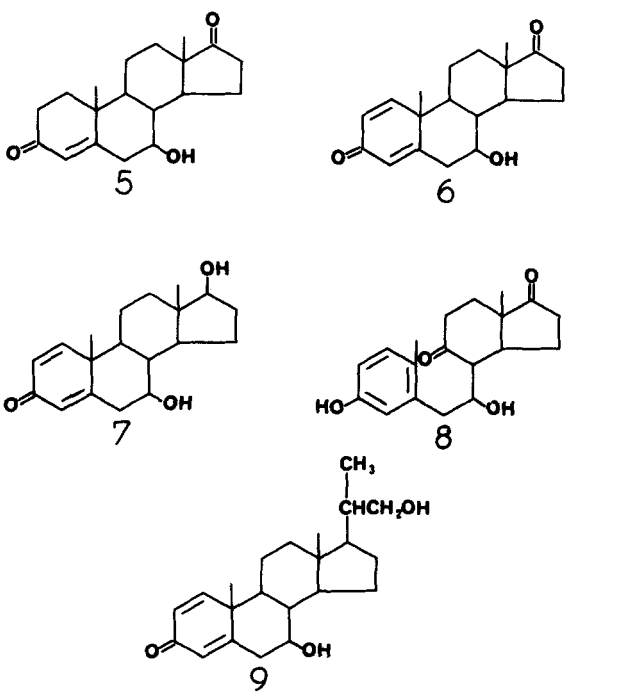

Fig. 4. Neutral metabolites isolated after the aerobic biotransformation of ursodeoxycholic acid by *Pseudomonas* sp NCIB 10590. 5: 7β-Hydroxyandrost-4-ene-3,17-dione; 6: 7β-hydroxyandrosta-1,4-diene-3,17-dione; 7: 7β,17β-dihydroxyandrosta-1,4-diene-3-one; 8: 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione-7β-ol; 9: 2α-hydroxymethylpregna-1,4-diene-3-one-7β-ol.

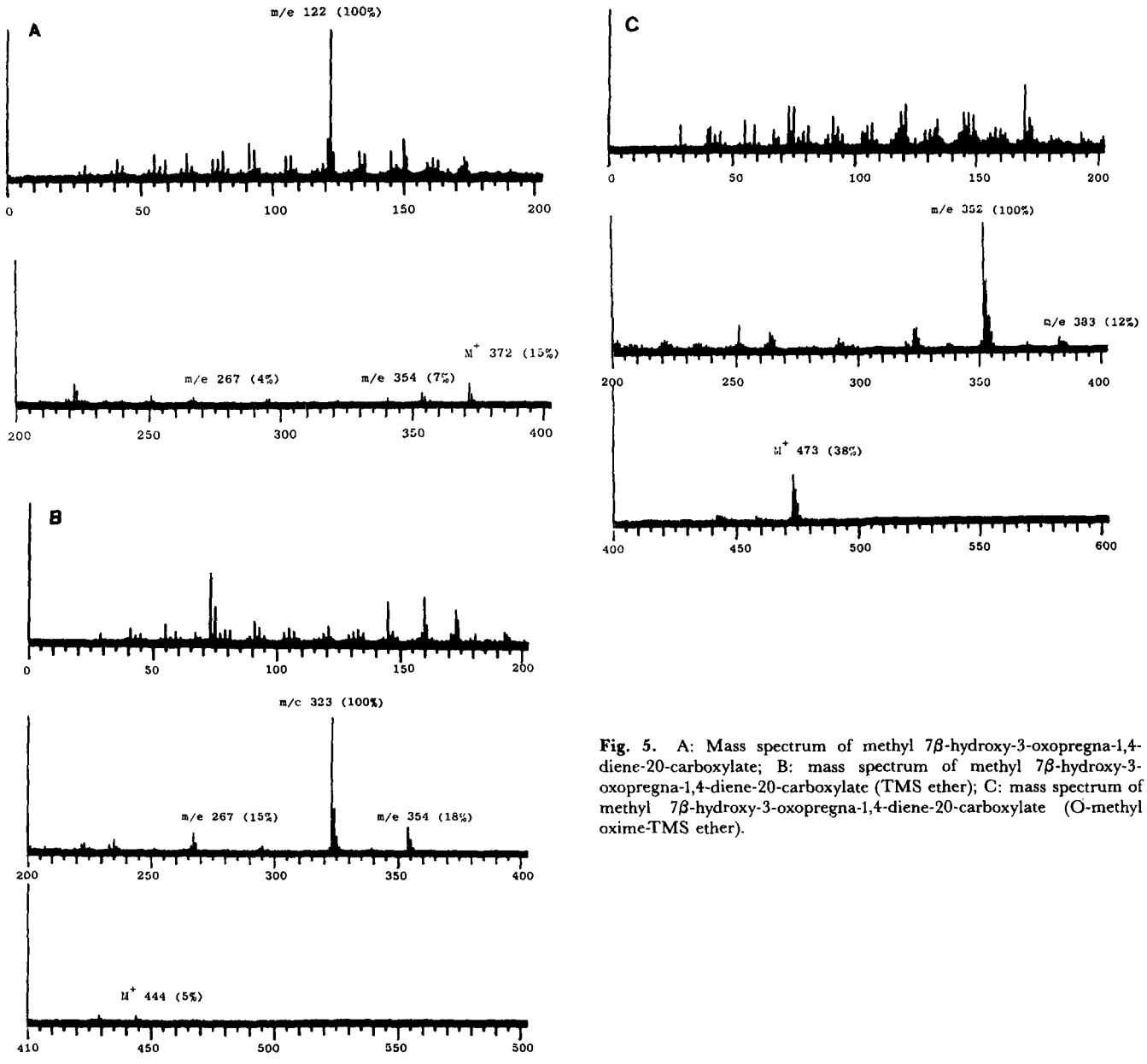

Fig. 5. A: Mass spectrum of methyl 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylate; B: mass spectrum of methyl 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylate (TMS ether); C: mass spectrum of methyl 7β-hydroxy-3-oxopregna-1,4-diene-20-carboxylate (O-methyl oxime-TMS ether).

hydroxyl group was assigned the 7β-configuration and metabolite 6 the structure 7β-hydroxyandrosta-1,4-diene 3,17-dione.

The mass spectrum of metabolite 5 showed a base peak molecular ion at m/e 302 (M^+, 100%) and an intense ion at m/e 124 (72%) that is typical for a steroidal 4-ene-3-one A-ring structure (19). Confirmation of this A-ring structure was provided by the UV spectrum (λ max, 242 nm, di-β-substituted αβ-unsaturated ketone in a six-membered ring, double bond exocyclic), IR spectrum (1660 and 1610 cm^{-1}, αβ-unsaturated ketone). Reactions on TLC revealed that metabolite 5 possessed a hydroxyl group and an unconjugated ketone group. This was confirmed by the IR spectrum and the mass spectrum of the O-methyl oxime-TMS ether which gave a base peak molecular ion at m/e 432 (M^+, 100%) consistent with the addition of TMS to a hydroxyl group and O-methyl oxime to two distinct ketone groups (3-ketone plus one unconjugated ketone group). The NMR spectrum of metabolite 5 displayed a broad multiplet centered at 3.50 δ and on this basis the hydroxyl group was assigned the 7β-configuration and metabolite 5 the structure 7β-hydroxyandrost-4-ene-3,17-dione.

Three minor neutral components were isolated from the neutral fraction. The UV spectrum of metabolite 7 (λ max, 244 nm, di-β-substituted αβ-unsaturated ketone in a six-membered ring, double bond exocyclic) was characteristic of a steroidal 1,4-diene-3-one A-ring structure (19).

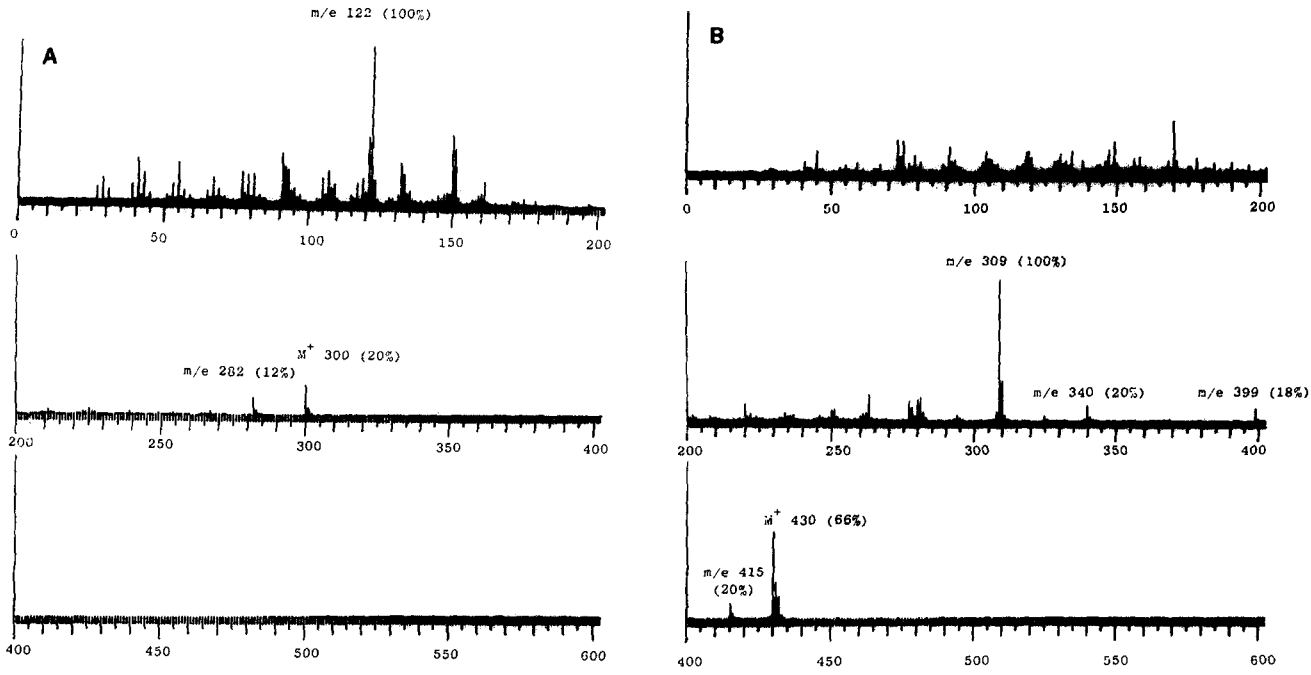

Fig. 6. A: Mass spectrum of 7β-hydroxyandrosta-1,4-diene-3,17-dione; B: mass spectrum of 7β-hydroxyandrosta-1,4-diene-3,17-dione (O-methyl oxime-TMS ether).

This was confirmed by the IR spectrum; 1739, 1610 and 1600 cm^{-1} ($\alpha\beta$-unsaturated ketone) and by the NMR spectrum (three vinylic protons in the range 5.94–6.78 δ). Metabolite 7 was oxidized to a compound of identical R_{fADD} to the oxidation product of metabolite 6. Metabolite 7 was readily acetylated but could not be reduced on TLC and was therefore classified as a 7,17-diol. This was confirmed by the IR spectrum which displayed two significant peaks (3350 and 3330 cm^{-1}) in the hydroxyl group region. The mass spectrum of the TMS-ether derivative displayed a molecular ion at m/e 446 (M^+, 11%) with significant ions at m/e 356 (M^+ – 90, TMS, 33%) and m/e 266 (M^+ – 180, 2TMS, 23%) consistent with the presence of two hydroxyl groups. This was confirmed by the mass spectrum of the O-methyl oxime-TMS ether derivative. The position and stereochemistry of the hydroxyl groups in metabolite 7 were assigned from the NMR spectrum; the presence of a triplet centered at 3.42δ and a broad multiplet centered at 3.62δ being consistent with the presence of a 17β- and a 7β-hydroxyl group, respectively. Metabolite 7 was therefore assigned the structure 7β,17β-dihydroxyandrosta-1,4-diene-3-one.

One of the remaining neutral compounds, 8, was recognized as a phenolic compound from its UV spectrum, λ max, 283 nm which underwent a bathochromic shift on addition of base to λ max, 296 nm. The shift was reversed on addition of acid. When sprayed with Folin-Ciocalteau reagent on a TLC plate and developed in an atmosphere of ammonia, the compound gave a characteristic blue color which is specific for phenolic hydroxyl groups (22). The mass spectrum of the TMS ether-derivative gave a molecular ion at m/e 460 (M^+, 13%) indicating the presence of two hydroxyl groups. This was corroborated by the mass spectrum of the O-methyl oxime-TMS ether derivative which displayed a molecular ion at m/e 518 (M^+, 55%) which was consistent with the presence of two hydroxyl groups and two ketone groups. Metabolite 8 was easily acetylated, oxidized, and reduced indicating the presence of hydroxy groups and unconjugated ketone groups. This was confirmed by the IR spectrum which showed two peaks consistent with a D-ring ketone (1730 cm^{-1}) and a C-ring ketone (1700 cm^{-1}) and a broad peak corresponding to hydroxy functions (3465 cm^{-1}). The IR spectrum also suggested a phenolic structure to this metabolite with peaks at 1615, 1585, and 1500 (aromatic ring), 1285, 1255 (phenol), and at 850 and 810 cm^{-1} (*o,m*-substituted phenol ring). The NMR spectrum indicated that one of the hydroxyl groups present on the molecule was in the 7β-configuration. Metabolite 8 has therefore been assigned the structure 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene 9,17-dione-7β-ol.

The mass spectrum of the final neutral product, metabolite 9, showed an intense ion (base peak) at m/e 122 (100%) that is typical for a steroidal 1,4-diene-3-one A-ring structure. This was confirmed by the UV spectrum (λ max, 245 nm, di-β-substituted $\alpha\beta$-unsaturated ketone

in a six-membered ring, double bond exocyclic), the IR spectrum (1660, 1610 and 1600 cm^{-1}, $\alpha\beta$-unsaturated ketone) and NMR spectrum (three vinylic protons in the range 6.07–7.06δ).

Metabolite 9 was easily oxidized and acetylated but could not be reduced on TLC. This suggested the presence of a hydroxy group and the lack of an unconjugated ketone group. This was confirmed by the IR spectrum which displayed a significant broad peak (3440 cm^{-1}) in the hydroxyl group region.

Metabolite 9, thus, in all respects appeared neutral and the lack of a free ketone group at C_{17} indicated the presence of an alkyl side chain. The mass spectrum showed a molecular ion at m/e 344 (M^+, 36%) consistent with the possession of a 3-carbon side chain. The mass spectrum of the TMS-ether derivative displayed a molecular ion at m/e 488 (M^+, 11%) with low intensity ions at m/e 398 (M^+ – 90, TMS, 11%) and m/e 308 (M^+ – 180, 2TMS, 5%) suggesting the presence of two hydroxyl groups. Furthermore, the spectrum also confirmed the possession of a 3-carbon side chain on the molecule with a significant ion at m/e 267 (M^+ – 221, 3C-side chain + 2TMS, 33%).

The position and stereochemistry of the hydroxyl groups was deduced from the NMR spectrum. The presence of a broad multiplet centered at 3.50δ, according with the presence of a 7β-hydroxyl group, while a singlet at 1.90δ and double doublets at 3.10 and 3.34 were consistent with a hydroxy group at C_{22}.

Metabolite 9 has therefore been assigned the structure 22α-hydroxymethylpregna-1,4-diene-3-one-7β-ol.

DISCUSSION

The isolation and identification of the steroidal metabolites 4–9 from the biotransformation of UDCA by *Pseudomonas* sp 10590 is the first recorded evidence of the microbial side-chain cleavage of this bile acid. Indeed metabolites 3–9 are novel steroids.

The biotransformation of UDCA by *Pseudomonas* sp 10590 has some similarities to the pattern described for the metabolism of CDCA (23). 1,4-Diene-3-oxo steroids reached a maximum at 15 hr compared to 14 hr for CDCA. However, while the production of major acidic metabolites was very similar for both UDCA (0.61 mM) and CDCA (0.50 mM), the major neutral metabolites only represented 0.1 mM for UDCA versus 0.6 mM for CDCA. Additionally, unsaturated C_{24} and C_{22} acidic metabolites were detected during the metabolism of UDCA, but only C_{22} acidic metabolites were detected during the metabolism of CDCA. The probable reasons for these differences are that the β-orientated 7-hydroxyl group of UDCA is slightly inhibitory to side-chain cleavage and conversely less inhibitory to 9-hydroxylation allowing ring fission to proceed more rapidly. These conclusions are supported by the accumulation of metabolite 3 during the metabolism of UDCA and that complete metabolism of UDCA occurs in 20 hr as opposed to 24 hr for CDCA (23).

The first product to be detected in the medium was metabolite 2 (2 hr) indicating that 3α-hydroxysteroid dehydrogenase is the initial enzyme to be induced. The next enzyme to be induced is the Δ^4-nuclear steroid dehydrogenase as envinced by the appearance of metabolite 3 in the culture medium (4 hr). At 6 hr metabolite 4 was detected indicating that unsaturated A-ring steroids are substrates for side-chain cleavage enzymes. Androstanes (metabolites 5–7) were not detected until after 10 hr incubation with the secosteroid (metabolite 8) appearing for the first time at 14 hr. Mechanisms of secosteroid formation have been discussed previously (24) and involves 9-hydroxylation of androstanes followed by a reverse *trans*-aldol rearrangement to form secosteroids. The presence of a 7β-hydroxyl group on UDCA apparently does not interfere with these reactions. The production of secosteroids enables bacteria to sequentially degrade the bile acid nucleus in a manner previously described (25). An additional new compound, 22α-hydroxymethylpregna-1,4-diene-3-one-7β-ol was also isolated in these experiments and it is believed to represent a side reaction and not an intermediate in the major pathway leading to the production of androstanes from UDCA.

In conclusion, *Pseudomonas* sp 10590 is capable of utilizing UDCA as sole carbon source and in the process a number of novel unsaturated C_{24}, C_{22}, and C_{19} intermediates are produced. The transformation of UDCA by *Pseudomonas* sp 10590 is very different from that of the colonic bacteria (26) and it is unlikely that metabolites 3–9 can be formed in the intestine. Nevertheless, the novel intermediates isolated are being screened for mutagenic activity and if they are non-mutagenic their pharmacological potential can be assessed.

We are extremely grateful to the Cancer Research Campaign for providing equipment which enabled mass spectral analyses to be conducted.

Manuscript received 3 April 1987 and in revised form 5 November 1987.

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