Biotransformation of chenodeoxycholic acid by *Pseudomonas* species NCIB 10590 under anaerobic conditions

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Abstract The metabolism of chenodeoxycholic acid by *Pseudomonas* sp. NCIB 10590 under strict anaerobic conditions was studied. A range of unsaturated acidic and neutral metabolites were isolated and identified. The major acidic product was chola-4,6-dien-3-one-24-oic acid whilst the major neutral product was androsta-4,6-dien-3,17-dione. The major acidic products were 7α-hydroxy-5β-cholan-3-oxo-24-oic acid, 3-oxo-4,6-pregnadien-20-carboxylic acid, 7α-hydroxy-3-oxo-1,4-pregnadien-20-carboxylic acid, and 7α-hydroxy-3-oxo-4-pregnen-20-carboxylic acid. The minor neutral products were androsta-1,4,6-trien-3,17-dione, 17β-hydroxyandrosta-4,6-dien-3,17-dione, 17β-hydroxyandrosta-1,4,6-trien-3-one, 7α-hydroxyandrosta-1,4-dien-3,17-dione, and 7α-hydroxyandrost-4-en-3,17-dione. In contrast to aerobic catabolism of chenodeoxycholic acid by *Pseudomonas* sp. NCIB 10590 in which 1,4-dienone steroids predominate, the major products described in this study are 4,6-dienone steroids. This is because of the induction of a 7α-dehydroxylase enzyme under anaerobic conditions.—**Owen, R. W., M. J. Hill, and R. F. Bilton.** Biotransformation of chenodeoxycholic acid by *Pseudomonas* species NCIB 10590 under anaerobic conditions. *J. Lipid Res.* 1983. **24:** 1109–1118.

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The transformation of bile acids by bacteria has generated a great deal of interest in recent years especially with respect to diseases of the human gastrointestinal tract (1, 2). Additionally, bile acid biotransformation products are potentially of importance in the commercial production of physiologically active steroids (3).

There are many reports of chenodeoxycholic acid (CDCA) metabolism by bacteria and these refer mainly to dehydroxylation and reversible oxidation of hydroxyl groups (4, 5). 7α-Dehydroxylation of CDCA is a predominant reaction carried out by intestinal bacteria yielding lithocholic acid as a major bile acid in feces.

However, there are only a few reports of nuclear desaturation and side-chain cleavage of CDCA by bacteria. Tenneson et al. (6) have shown that *Pseudomonas* sp. NCIB 10590 has the ability to degrade CDCA under aerobic conditions yielding 7α-hydroxy-3-oxo-1,4-pregnadien-20-carboxylic acid and 7α-hydroxy-1,4-androstadiene-3,17-dione as major products. Similar products were obtained from CDCA with *E. coli* grown under anaerobic conditions (7).

In this communication evidence is presented for the extensive degradation of CDCA by anaerobic resting cultures of *Pseudomonas* sp. NCIB 10590.

MATERIALS

CDCA, 5α-cholestane, and androsta-1,4-dien-3,17-dione were obtained from Koch Light Laboratories (Colnbrook, Bucks). General reagents were of Analar grade and obtained from BDH. All solvents were redistilled before use.

METHODS

Melting points (mp) were determined using a Kofler hot-stage 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 Perkin-Elmer 457 spectrophotometer. Nuclear magnetic resonance (NMR) proton spectra were recorded on a Jeol 220 spectrometer operating at 220 MHz at 30°C from solutions in deuterated chloroform. Mass spectra (MS) were obtained using a Dupont 21-491 series mass

Abbreviations: FBA, fecal bile acid; CDCA, chenodeoxycholic acid; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; MS, mass spectra; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; ADD, androsta-1,4-dien-3,17-dione.

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spectrometer either by direct inlet, or by combined GC–mass spectrometry using a Varian aerograph 2700 gas chromatograph. Data reduction was performed with a Dupont 21-0948 data system.

Gas–liquid chromatography (GLC)

Analysis by GLC was performed at 260°C using 3% OV-17 on 80/100 mesh acid-washed silanized Supelcoport in 1.5 m × 3 mm silanized glass columns obtained from Phase Separations (Queensferry, UK). Retention times were measured relative to the internal standard 5α-cholestane with a flow rate of 30 ml min^{-1} nitrogen through the column and 30 ml min^{-1} H_2 to the flame ionization detector in a Hewlett-Packard H.P. 5470 instrument.

Thin-layer chromatography (TLC)

Analysis by TLC was performed on 0.25-mm layers of Kieselgel GF_{254} (obtained from E. Merck, Darmstadt, West Germany) in methanol–dichloromethane 1:19 (v/v) and the mobilities were measured relative to androsta-1,4-dien-3,17-dione (ADD). Products containing a 4-en-3-one, 1,4-dien-3-one, 4,6-dien-3-one, and 1,4,6-trien-3-one chromophore were detected under ultraviolet light (254 nm) and all components were finally visualized as characteristically colored spots by spraying the plates with anisaldehyde reagent (8) and heating in an oven at 110°C for 10 min.

As an aid to tentative identification of steroids by TLC, oxidation, acetylation, and reduction were carried out as described by Tenneson et al. (9). Acidic steroids were methylated with ethereal diazomethane (10) prior to analysis.

The biotransformation medium

The culture medium contained (g/l of distilled water) sodium chenodeoxycholate, 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, 7.2. Solutions of sodium chenodeoxycholate, mineral salts, trace elements, and magnesium sulfate were autoclaved separately prior to admixture. Shake-flask cultures were grown on an L. H. Engineering orbital incubator at 28°C. Culture aliquots were checked by TLC and GLC to ensure that carryover of aerobic metabolites did not occur.

The fermentation

Cells obtained from eight 1-liter shake-flask cultures of *Pseudomonas* sp. NCIB 10590 by centrifugation at 10,000 *g* on an MSE Mistral 4L centrifuge were used to inoculate 8 liters of the culture medium. This gave a resting cell suspension of the Pseudomonad with a final cell density of 1×10^9 cells ml^{-1} (OD of 1.0 at 540 nm). The culture medium was steamed for 1 hr immediately before inoculation to remove dissolved oxygen. The top of the fermentation bottle was loosened (to allow gaseous exchange) prior to incubation at 28°C for 6 weeks under 90% H_2–10% CO_2 (with palladium catalyst to remove residual oxygen). The course of the fermentation and OD_{540} was followed at weekly intervals. After incubation the fermentation was terminated by direct extraction of the metabolites into an equal volume of ethyl acetate. After drying over anhydrous $MgSO_4$, the solvent was removed in vacuo at 30°C to yield 4 g of residue. The residue was separated into acidic (3.1 g) and neutral (910 mg) fractions. The acidic fraction was methylated using boron trifluoride–methanol and separated by preparative TLC (~100 mg of crude extract per plate) in the solvent system methanol–dichloromethane 1:19 (v/v) yielding residual CDCA (950 mg) and a series of fractions from which steroids 8–12 were crystallized. The neutral fraction was separated likewise giving steroids 2–7.

Androsta-4,6-dien-3,17-dione (*2*)

Recrystallization of *2* (**Fig. 1**) from methanol–dichloromethane gave yellow needles (152 mg); mp 172–173°C; IR, 1746 (17-ketone), 1664 (3-ketone), 1616 and 1582 cm^{-1} (C_4–C_5 and C_6–C_7 double bonds); UV, 284 nm (ξ 28,350); NMR, (δ) 0.97, 1.15 (6H, s, 18-CH_3 and 19-CH_3), 2.35–2.65 (2H, m, 16-CH_2), 5.73 (1H, s, 4-H), 6.20 (2H, s, 6-H and 7-H); MS, M^+ 284 (74%, $C_{19}H_{24}O_2$ requires M^+, 284), m/e 136 (4,6-diene-3-one, 100%); GLC, R_f 1.50; TLC, R_f 1.08; after oxidation, R_f 1.08; after acetylation, R_f 1.08; and after reduction R_f 0.75.

Androsta-1,4,6-trien-3-17-dione (*3*)

Recrystallization of *3* (Fig. 1) from methanol–dichloromethane gave white crystals (92 mg); mp 164–167°C; IR, 1746 (17-ketone), 1656 (3-ketone), 1632, 1606, and 1586 cm^{-1} (C_1–C_2, C_4–C_5, and C_6–C_7 double bonds); UV, 224, 256, and 300 nm (ξ 12,740, 10,500, and 13,670); NMR, (δ) 1.00, 1.23 (6H, s, 18-CH_3 and 19-CH_3) 2.40–2.65 (2H, M, 16-CH_2), 6.06–6.37 (4H, m 2-H, 4-H, 5-H, and 7-H), 7.07 (1H, d, J = 10 Hz, 1-H); MS, M^+ 282 (96%, $C_{19}H_{22}O_2$ requires M^+ 282), and m/e 134 (1,4,6-trien-3-one, 100%); GLC, R_f 1.60; TLC, R_f 1.03; after oxidation, R_f 1.03; after acetylation, R_f 1.03; and after reduction, R_f 0.68.

17β-Hydroxyandrosta-4,6-dien-3-one (*4*)

Recrystallization of *4* (Fig. 1) from methanol–dichloromethane gave yellow spiky needles (80 mg); mp 198–200°C; IR, 3405 (hydroxyl), 1656 (3-ketone), 1620 and 1586 cm^{-1} (C_4–C_5 and C_6–C_7 double bonds); UV, 285 nm (ξ 22,017); NMR, (δ) 0.83, 1.12 (6H, s, 18-CH_3 and

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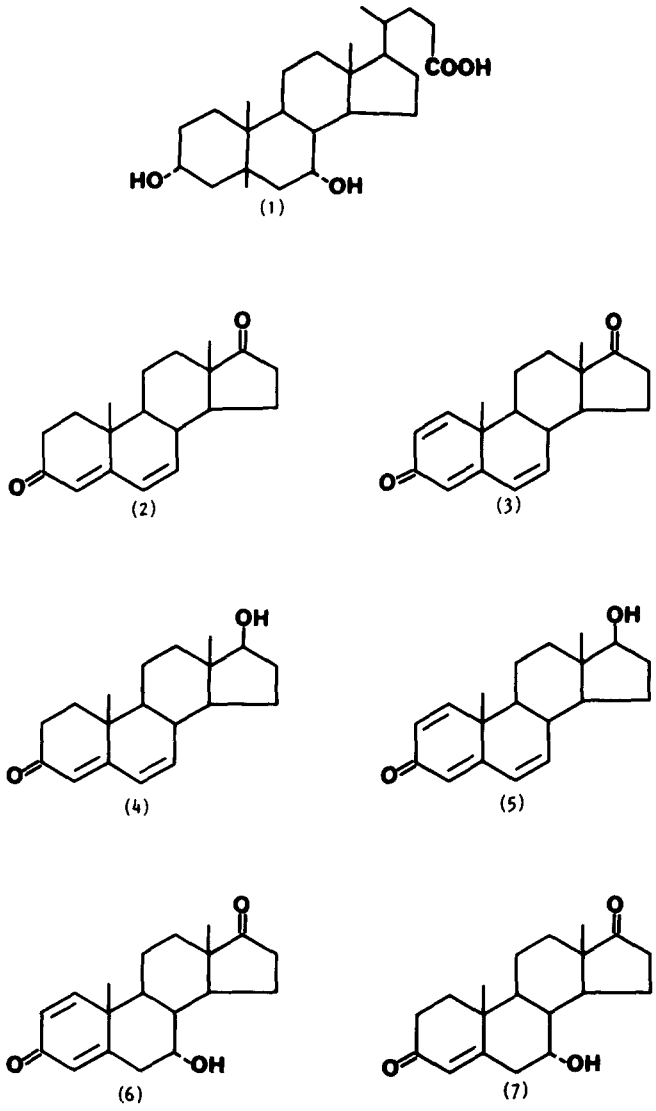

Fig. 1. Neutral metabolites isolated after the anaerobic degradation of chenodeoxycholic acid by *Pseudomonas* sp. NCIB 10590.

19-CH_3), 3.68 (1H, t, J = 8 Hz, 17-H), 5.66 (1H, s, 4-H) 6.09 (2H, s, 6-H and 7-H); MS, M^+ 286 (100%, $C_{19}H_{26}O_2$ requires M^+ 286) intense ion at m/e 136 (4,6-dien-3-one, 94%) and low intensity ion at m/e 268 (M^+-18, H_2O, 45%); GLC, R_f 1.58; TLC, R_f 0.75; after oxidation, R_f 1.08; after acetylation, R_f 1.15; and after reduction, R_f 0.75.

17β-Hydroxyandrosta-1,4,6-trien-3-one (*5*)

Recrystallization of *5* (Fig. 1) from methanol–dichloromethane gave white needles (108 mg); mp 150–153°C; IR, 3435 (hydroxyl), 1661 (3-ketone), 1650, 1612, and 1586 cm^{-1} (C_1–C_2, C_4–C_5, and C_6–C_7 double bonds); UV, 224, 256, 300 nm (ξ 13,000, 12,250, and 14,980); NMR, (δ) 0.95, 1.14 (6H, s, 18-CH_3 and 19-CH_3), 3.68 (1H, t, J = 8 Hz, 17-H), 6.04–6.36 (4H, m, 2-H, 4-H, 6-H, and 7-H), 7.05 (1H, d, J = 10 Hz, 1H); MS, M^+ 284 (84%, $C_{19}H_{24}O_2$ requires M^+ 284), base peak at m/e 134 (1,4,6-trien-3-one, 100%) and low intensity ion at m/e 266 (M^+-18, H_2O, 32%); GLC, R_f 1.50; TLC, R_f 0.68; after oxidation, R_f 1.03; after acetylation, R_f 1.09; and after reduction, R_f 0.68.

7α-Hydroxyandrosta-1,4-dien-3,17-dione (*6*)

Recrystallization of *6* (Fig. 1) from methanol–dichloromethane gave white prisms (96 mg); mp 238–239°C; IR, 3330 (hydroxyl), 1738 (17-ketone), 1658 (3-ketone), 1610 and 1596 (C_1—C_2 and C_4–C_5 double bonds); UV, 244 nm (ξ 14,960); NMR, (δ) 0.95, 1.26 (6H, s, 18-CH_3 and 19-CH_3), 2.40–2.58 (2H, m, 16-CH_2), 2.79 (1H, s, 7-H), 6.17 (1H, s, 4-H), 6.27 (1H, d, showing further splitting, J = 10 Hz, 2-H), 7.07 (1H, d, J = 10 Hz, 1-H); MS, M^+ 300 (19%, $C_{19}H_{24}O_3$ requires M^+ 300), base peak at m/e 122 (1,4-dien-3-one, 100%) and intense ion at m/e 282 (M^+-18, H_2O, 31%); GLC, R_f 2.90; TLC, R_f 0.52; after oxidation, R_f 1.01; after acetylation, R_f 1.01; and after reduction, R_f 0.28.

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

Recrystallization of *7* (Fig. 1) from methanol–dichloromethane gave white powdery crystals (72 mg); mp 225–226°C; IR, 3340 (hydroxyl), 1740 (17-ketone), 1660 (3-ketone), and 1610 cm^{-1} (C_4–C_5 double bond); UV, 240 nm (ξ 16,500); MS, M^+ 302 (30%, $C_{19}H_{26}O_2$ requires M^+ 302), base peak at m/e 124 (4-en-3-one, 100%) and low intensity ion at m/e 284 (M^+-18, H_2O, 22%); GLC, R_f 2.50; TLC, R_f 0.54; after oxidation, R_f 1.03; after acetylation, R_f 1.05; and after reduction, R_f 0.35.

Methyl 7α-hydroxy-5-cholan-3-oxo-24-oate (methyl ester of *8*)

Recrystallization of methyl *8* (**Fig. 2**) from diethyl ether gave white spiky crystals (251 mg); mp 125–127°C; IR, 3340 (hydroxyl), 1730 (carboxyl), and 1710 (3-ketone); UV, 284 nm (ξ 110); NMR, (δ) 0.70, 1.10 (6H, s, 18-CH_3 and 19-CH_3), 0.94 (3H, s, 21-CH_3), 3.68 (3H, s, 24-OCH_3) and 5.78 (1H, t, J = 3 Hz, 7-H; MS, M^+ 404 (26%, $C_{25}H_{40}O_4$ requires M^+ 404), low intensity ion at m/e 386 (M^+-18, H_2O, 40%) and base peak at m/e 271 (M^+-133 6C-side chain + H_2O, 100%); GLC, R_f 9.30; TLC, R_f 0.92; after oxidation, R_f 1.13; after acetylation, R_f 1.18; and after reduction, R_f 0.44.

Methyl chola-4,6-dien-3-one-24-oate (methyl ester of *9*)

Recrystallization of methyl *9* (Fig. 2) from diethyl ether gave yellow needles (502 mg); mp 155°C; IR, 1738 (carboxyl), 1666 (3-ketone), 1618 and 1586 cm^{-1} (C_4–C_5 and C_6–C_7 double bonds); UV, 284 nm (ξ

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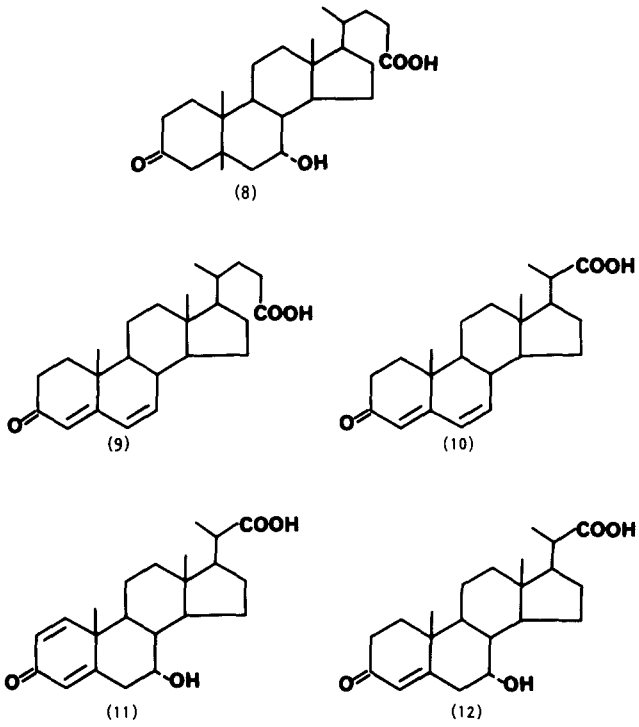

Fig. 2. Acidic metabolites isolated after the anaerobic degradation of chenodeoxycholic acid by *Pseudomonas* sp. NCIB 10590.

27,580); NMR, (δ) 0.76, 1.11 (6H, s, 18-CH_3 and 19-CH_3), 0.93 (3H, d, J = 5 Hz, 21-CH_3), 3.68 (3H, s, 24-OCH_3), 5.67 (1H, s, 4-H); MS, M^+ 384 (100%, $C_{25}H_{36}O_3$ requires M^+ 384), intense ion at m/e 136 (4,6-dien-3-one, 72%) and intense ion at m/e 269 (M^+-115, 6C-side chain, 51%); GLC, R_f 7.00; TLC, R_f 1.14; after oxidation, R_f 1.14; after acetylation, R_f 1.14; and after reduction, R_f 1.14.

Methyl 3-oxo-4,6-pregnadien-20-oate (methyl ester of *10*)

Recrystallization of methyl *10* (Fig. 2) from diethyl ether gave a slightly impure solid (30 mg); IR, 1744 (carboxyl), 1666 (3-ketone), 1616 and 1590 cm^{-1} (C_4–C_5 and C_6–C_7 double bonds); UV, 284 nm (28,460); MS, M^+ 356 (38%, C_{23} $H_{32}O_3$ requires M^+ 356), intense ion at m/e 136 (4,6-dien-3-one, 54%) and low intensity ion at m/e 269 (M^+-87, 4C-side chain, 15%); GLC, R_f 3.40; TLC, R_f 1.12; after oxidation, R_f 1.12; after acetylation, R_f 1.12; and after reduction, R_f 1.12.

Methyl 7α-hydroxy-3-oxo-1,4-pregnadien-20-oate (methyl ester of *11*)

Recrystallization of methyl *11* (Fig. 2) from diethyl ether gave white prisms (63 mg); mp 259–260°C; IR, 3535 (hydroxyl), 1720 (carboxyl), 1652 (3-ketone), 1612 and 1600 cm^{-1} (C_1–C_2 and C_4–C_5 double bonds); UV, 244 nm (ξ 15,000); MS, M^+ 372 (5% $C_{23}H_{32}O_4$ requires M^+ 372), base peak at m/e 122 (1,4-dien-3-one, 100%), low intensity ion at m/e 354 (M^+-18, H_2O, 24%) and low intensity ion at m/e 267 (M^+-105, 4C-side chain + H_2O, 16%); GLC, R_f 7.40; TLC, R_f 0.44; after oxidation, R_f 1.07; after acetylation, R_f 1.08; and after reduction, R_f 0.44.

Methyl 7α-hydroxy-3-oxo-4-pregnen-20-oate (methyl ester of *12*)

Recrystallization of methyl *12* (Fig. 2) from diethyl ether gave white spiky needles (40 mg); mp 252–253°C; IR, 3530 (hydroxyl), 1730 (carboxyl), 1650 (3-ketone), and 1610 (C_4–C_5 double bonds); UV, 242 nm (ξ 14,600); MS, M^+ 374 (31%, C_{23} $H_{34}O_4$ requires M^+ 374), base peak at m/e 124 (4-en-3-one, 100%), intense ion at m/e 356 (M^+-18, H_2O, 94%) and low intensity ion at m/e 269 (M^+-105, 4C-side chain + H_2O, 33%); GLC, R_f 6.60; TLC, R_f 0.51; after oxidation, R_f 1.19; after acetylation, R_f 1.21; and after reduction, R_f 0.51.

RESULTS

CDCA was extensively degraded by a resting cell suspension of *Pseudomonas* sp. NCIB 10590 under anaerobic conditions. The cells remained viable throughout the experiment. Eleven metabolites (six neutral and five acidic) were isolated and the assigned structures are listed in Figs. 1 and 2. Unless otherwise stated, these metabolites were 100% pure.

The results described refer to one large scale experiment after pilot scale studies had revealed that the anaerobic catabolism of CDCA by *Pseudomonas* sp. NCIB 10590 was entirely reproducible.

The mass spectra of the major neutral metabolite *2* (**Fig. 3**) and its analogue *4* gave intense ions (base peaks) at m/e 136 that are typical for steroidal 4,6-dienone structures (11). Confirmation of this A,B-ring structure was provided by the UV spectra (λ max, 284 nm, di-β-substituted α,β-unsaturated ketone in a six-membered ring, double bond exocyclic, extended by a double bond at C_6–C_7) (12), IR spectra (1664, 1616, 1582 and 1656, 1620, 1586 cm^{-1}, $\alpha\beta$-unsaturated ketone) and PMR spectra (three vinylic protons in the range 5.66–6.20 δ).

Metabolite *2* resisted oxidation and acetylation but was reduced with potassium borohydride to a product that corresponded in $R_{f_{ADD}}$ and color, (purple mauve) to *4* on TLC. This indicated the lack of a hydroxyl group but the possession of an unconjugated ketone group. This was confirmed by the IR spectrum that did not display any peaks in the hydroxyl region but possessed a major peak at 1746 cm^{-1} (typical of an uncon-

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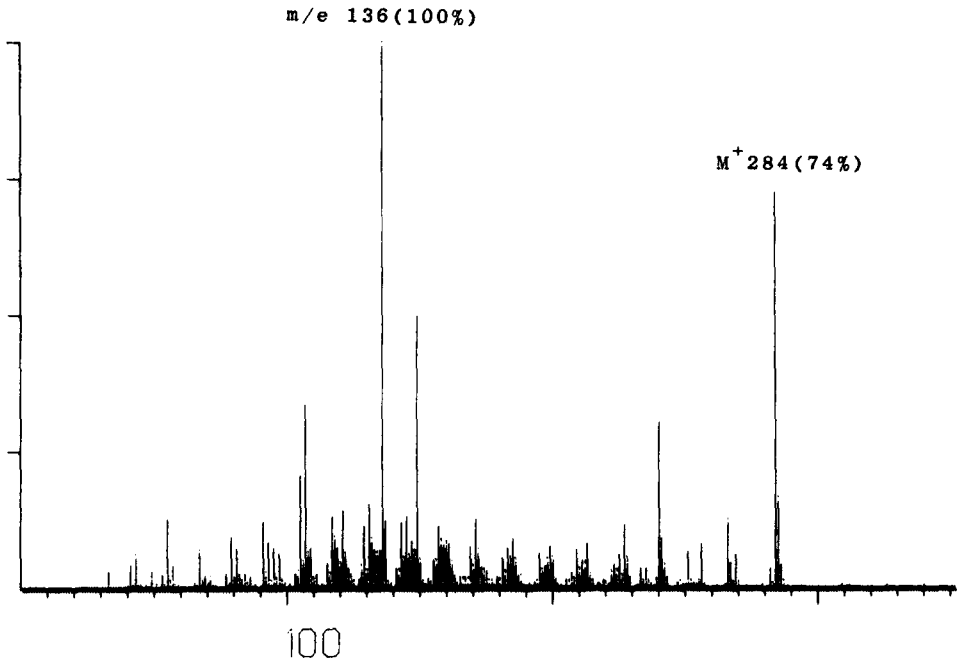

Fig. 3. Mass spectrum of androsta-4,6-dien-3,17-dione.

jugated ketone group adjacent to a five-membered ring).

The oxidation product of *4* corresponded with respect to $R_{f_{ADD}}$ and color (orange) to *2* on TLC. Metabolite *4* was easily acetylated but could not be reduced with borohydride, indicating the presence of a hydroxyl group and the lack of an unconjugated ketone group. This was confirmed by the IR spectrum that did not display a peak in the unconjugated carbonyl region but possessed a significant peak at 3405 cm^{-1} in the hydroxyl group region.

The position and stereochemistry of the hydroxyl group in *4* was assigned from the PMR spectrum. It has been shown that protons associated with carbon atoms carrying hydroxyl groups in certain steroids give rise to characteristic splitting patterns (13).

Compound *4* gave rise to a well-defined triplet at 3.68 δ which is characteristic for a 17α-proton (13). The 17α-proton presumably bisects the dihedral angle between the two protons at C_{16} with a coupling constant of 8 Hz. The hydroxyl group in *4* was thus assigned the 17β-configuration. Further support for this assignment is provided by the observation (14) that loss of the elements of water from the molecular ion in the mass spectrometer occurs less readily with β-equatorial hydroxyl groups than with α-axial hydroxyl groups. As a result the ion due to the loss of β-hydroxyl groups is less intense than the molecular ion, whilst the ion due to the loss of α-hydroxyl groups is more intense than the molecular ion. The mass spectrum of *4* displayed a low intensity ion at m/e 268 (45%) that was of lower intensity than the molecular ion M^+ 286 (100%). Compound *2* did not display any evidence of hydroxyl groups being present.

Compound *2* was therefore assigned the structure androsta-4,6-dien-3,17-dione and compound *4* was assigned the structure 17β-hydroxyandrosta-4,6-dien-3-one.

Compounds *3* and *5* also appeared from their mass spectral behavior to be structurally related. The mass spectra of *3* (**Fig. 4**) and *5* (**Fig. 5**) displayed intense ions (base peak) at m/e 134 indicating a 1,4,6-trien-3-one structure (11). Confirmation of this A,B-ring structure was provided by the UV spectra (λ max, 224, 256, 300 nm, di-β-substituted, α,β-unsaturated ketone, double bond exocyclic, extended by a double bond at C_6–C_7 and cross-conjugation (12)); IR spectra, 1656, 1632, 1606, 1586 and 1661, 1650, 1612, 1586 cm^{-1}, respectively, α,β-unsaturated ketone and PMR spectra, four vinylic protons in the range 6.06–6.37 δ.

Metabolite *3* resisted oxidation and acetylation but was reduced with borohydride to a product that corresponded in $R_{f_{ADD}}$ and color (blue) to *5* on TLC. This indicated the lack of a hydroxyl group and the possession of an unconjugated ketone group. This was confirmed by the IR spectrum. The oxidation product of *5* corresponded with respect to $R_{f_{ADD}}$ and color (mauve) to *3* on TLC. Compound *5* was also easily acetylated but could not be reduced with borohydride, indicating the presence of a hydroxyl group and the lack of an unconjugated ketone group. This was again confirmed by the IR spectrum.

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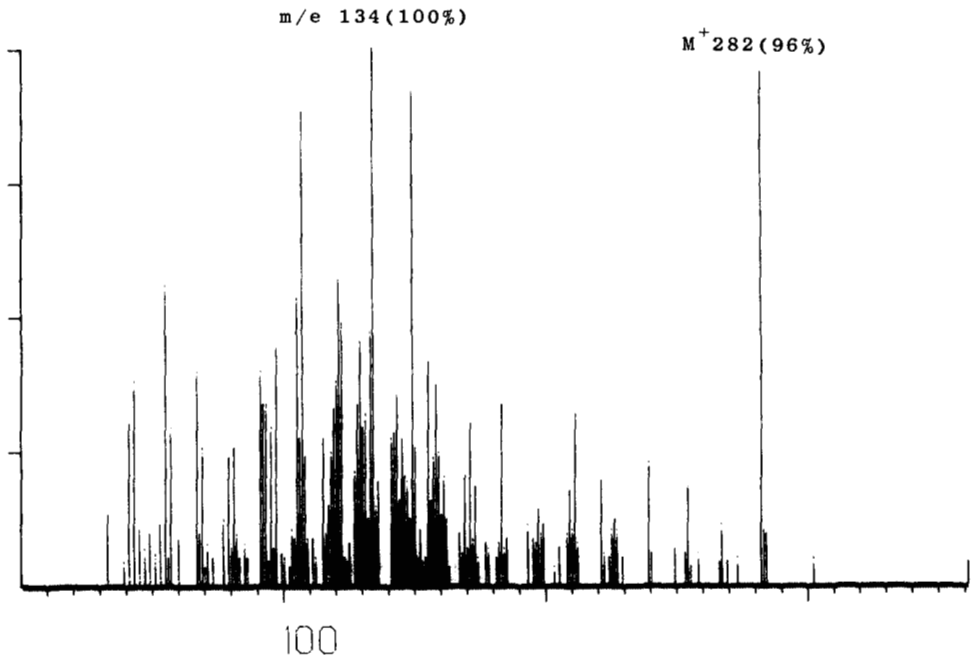

Fig. 4. Mass spectrum of androsta-1,4,6-trien-3,17-dione.

The PMR spectrum of *5* gave rise to four vinylic protons that exhibited a complex splitting pattern in the range 6.04–6.36 δ. The four protons could not be distinguished from one another but were taken as being typical of a 1,4,6-trien-3-one structure, since this is the first recorded evidence for a 1,4,6-trienone structure by PMR. Compound *5* also gave rise to a well-defined triplet centered at 3.68 δ which is characteristic for a 17α-proton. The hydroxyl group in *5* was thus assigned the 17β-configuration. The fragment ions of the mass spectrum supported this conclusion because a low intensity ion was evident at m/e 266 (32%) due to the loss of the elements of water from the molecular ion M^+ 284 (84%).

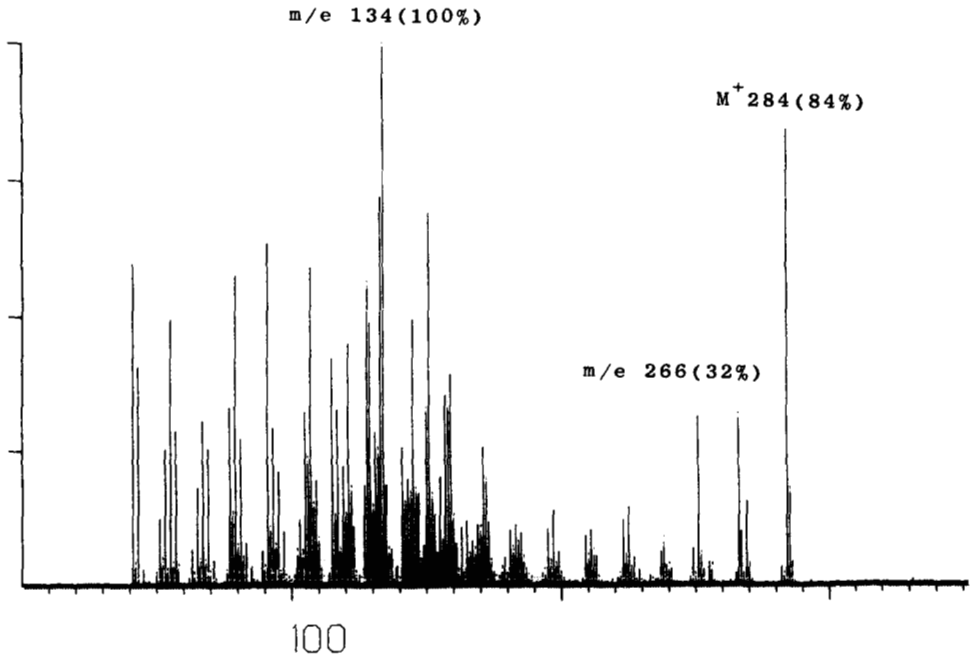

Fig. 5. Mass spectrum of 17β-hydroxyandrosta-1,4,6-trien-3-one.

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The PMR spectrum of *3* also gave rise to four vinylic protons which again exhibited a complex splitting pattern in the range 6.06–6.37 δ, substantiating a 1,4,6-trienone structure. However *3* did not display any evidence for a hydroxyl group in the PMR spectrum. This was confirmed by the mass spectrum which showed a molecular ion at m/e 282 (M^+, 100%) without a significant fragment ion at m/e 264.

Metabolite *3* was therefore assigned the structure androsta-1,4,6-trien-3,17-dione whilst metabolite *5* was assigned the structure 17β-hydroxyandrosta-1,4,6-trien-3-one.

Two other neutral compounds were isolated from the neutral fraction.

The mass spectrum of compound *6* showed a molecular ion at m/e 300 and an intense ion (base peak) at m/e 122, which is typical for a steroidal 1,4-dien-3-one, A-ring structure (15). Further spectroscopic analysis revealed it to be identical to 7α-hydroxyandrosta-1,4-dien-3,17-dione isolated by Tenneson et al. (6).

The mass spectrum (**Fig. 6**) of *7* showed a molecular ion at m/e 302 and an intense ion (base peak) at m/e 124 which is typical for a steroidal 4-en-3-one A-ring structure (15). Confirmation of this A-ring structure was provided by the UV spectrum (λ max, 242 nm di-β-substituted α,β-unsaturated ketone in a six-membered ring, double bond exocyclic), IR spectrum (1660 and 1610 cm^{-1}, α,β-unsaturated ketone). Reactions on TLC revealed that *7* possessed a hydroxyl group and an unconjugated ketone group. This was confirmed by the IR spectrum. Unfortunately the compound was not sufficiently pure to obtain a PMR spectrum and thus the position of the hydroxyl group was determined chemically.

Compound *7* was subjected to strong acid conditions at 55°C and this resulted in the production of a compound (100% conversion) that was identical to androsta-4,6-dien-3,17-dione in its TLC, GLC, and mass spectral properties. Because C_7 hydroxyl functions are subject to dehydration under the conditions used, the hydroxyl group in *7* was deemed to be at C_7. The stereochemistry of the hydroxyl group was inferred from the mass spectral analysis. Compound *7* gave rise to an intense ion at M^+ 18 (22%) with respect to the molecular ion (M^+, 30%) indicating the hydroxyl group at C_7 to be in the 7α-axial position (14). Compound *7* was therefore assigned the structure 7α-hydroxyandrost-4-en-3,17-dione.

Two major (*8* and *9*) and three minor (*10, 11,* and *12*) acidic intermediates were also isolated and structural determination was carried out on the methyl ester derivatives.

The methyl ester of compound *8* was found to be identical to methyl 7α-hydroxy-5β-cholan-3-oxo-24-oate synthesized by the method of Tserng (16) in its TLC and GLC behavior. Methyl *8* 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. The mass spectrum revealed a molecular ion at m/e 404 (M^+, 26%), an intense ion at M-18 (40%) with respect to the molecular ion, and a base peak at m/e 271 (100%) due to

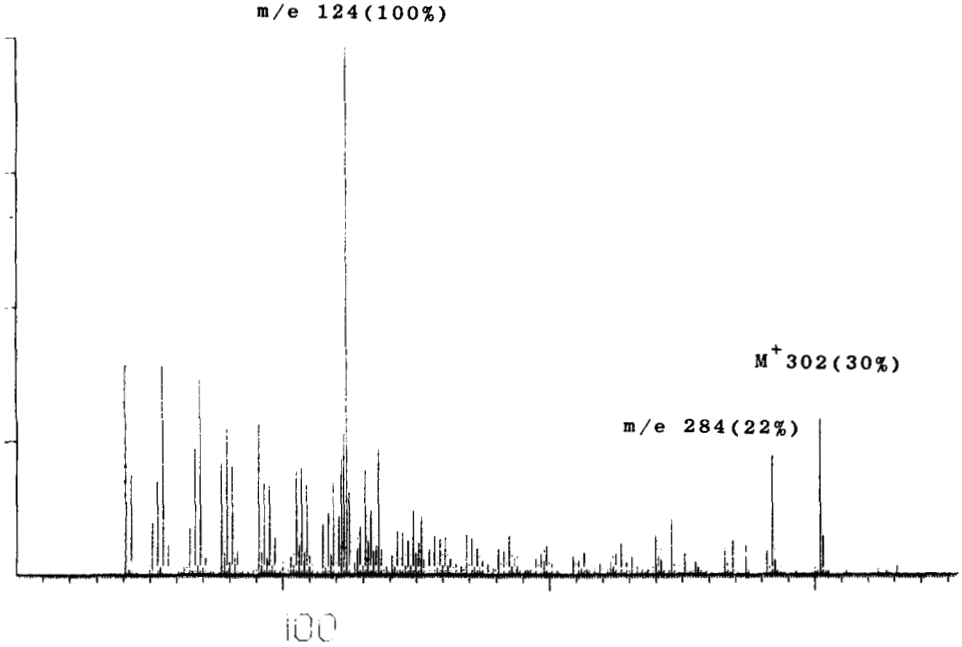

Fig. 6. Mass spectrum of 7α-hydroxyandrost-4-en-3,17-dione.

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the loss of the full bile acid side chain (i.e., 6C-side chain + H_2O). The PMR spectrum did not reveal any vinylic protons; however a triplet was present at 5.78 δ typical of a 7β-proton on a carbon carrying a 7α-hydroxyl group. The mass and PMR spectra indicated that the hydroxyl group in methyl *8* was of the 7α-axial configuration. Metabolite *8* was therefore assigned to the structure 7α-hydroxy-5β-cholan-3-oxo-24-oic acid.

Metabolites *9* and *10* appeared to be of a similar structure. The mass spectra of methyl *9* (**Fig. 7**) and methyl *10* both revealed intense ions at m/e 136 typical of 4,6-dien-3-oxo A,B-ring structures. This was supported by their UV spectra (λ max, 284 nm), IR spectra, and the PMR spectrum of methyl *9*. Reactions on TLC revealed that both metabolites did not possess any unconjugated ketone or hydroxyl groups. This was confirmed by the IR spectra. The PMR spectrum of *10* revealed three vinylic protons in the range 5.67–6.11 δ corroborating a 4,6-dienone A-ring structure. The mass spectrum of methyl *9* showed a base peak molecular ion at m/e 384 (M^+, 100%) and an intense ion at m/e 269 (51%) indicating the loss of the full bile acid side chain (15). However, the mass spectrum of methyl *10* revealed a low intensity molecular ion at m/e 356 (M^+, 38%) and a low intensity ion at m/e 269 (15%) due to the loss of a 4C-side chain (15). Compound *9* has therefore been assigned the structure chola-4,6-dien-3-one-24-oic acid and compound *10* the structure 3-oxo-4,6-pregnadien-20-carboxylic acid.

Compounds *11* and *12* were found to be identical in their TLC, GLC, and spectroscopic properties to those isolated by Tenneson et al. (6), namely 7α-hydroxy-3-oxo-1,4-pregnadien-20-carboxylic acid and 7α-hydroxy-3-oxo-4-pregnen-20-carboxylic acid. respectively.

The yield of steroidal metabolites isolated is listed in **Table 1.**

DISCUSSION

Under anaerobic conditions the catabolism of CDCA by *Pseudomonas* sp. NCIB 10590 gave 4,6-dien-3-oxo steroids (of which metabolites *2, 4, 5, 7, 9,* and *10* are novel bacterial metabolites) as the predominant intermediates. This contrasts markedly with the aerobic metabolism of the same substrate which gives 7α-hydroxy-1,4-dienone steroids as the major intermediates (6). The removal of the hydroxyl group at C_7 suggests that induction of a 7α-dehydroxylase enzyme occurs under anaerobic conditions.

It has been shown that the induction of 7α-dehydroxylase is strongly favored by anaerobic conditions (17) and the actual mechanism of the reaction has been studied in detail (18). The initial reaction in the 7α-dehydroxylation of cholic acid is a diaxial *trans* elimination of the 7α-hydroxyl group and the 6β-hydrogen atom yielding 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid. In the human intestine, where 7α-dehydroxylation of bile acids is very common (19), it has been shown that the Δ^6-acid is then reduced by *trans* hydrogenation at the 6α- and 7β-positions to give deoxycholic acid (19).

7α-Dehydroxylation, it appears, is not strictly confined to anaerobiosis, however, because it has been demonstrated that aerobically growing cultures of the soil microorganism, *Arthrobacter (Corynebacterium) simplex,*

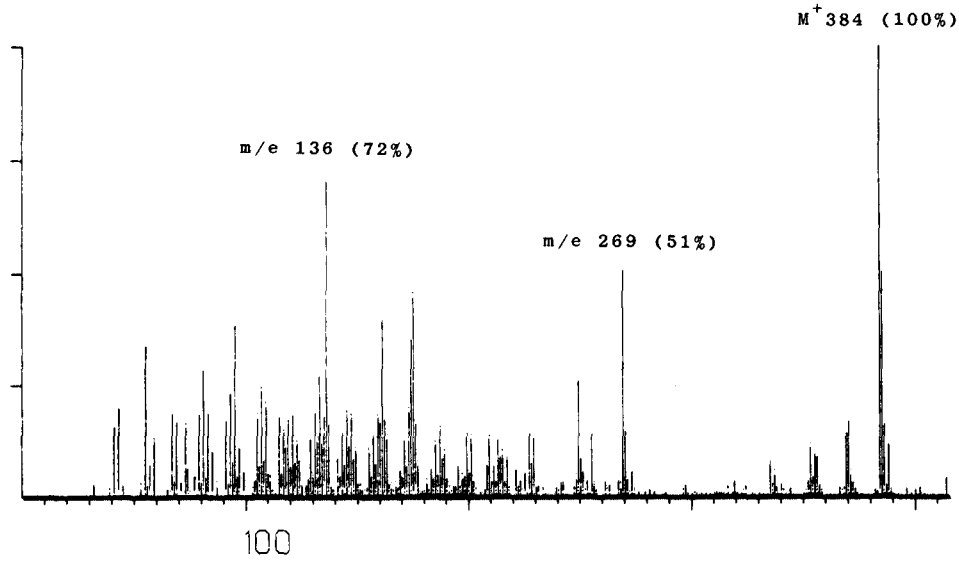

Fig. 7. Mass spectrum of chola-4,6-dien-3-one-24-oic acid.

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TABLE 1. Yield of metabolites with respect to starting material after 6 weeks anaerobic incubation

Metabolite Number	Metabolite	R_f[a]	Yield %	Yield mg
2	Androsta-4,6-dien-3,17-dione	1.08	1.37	152
3	Androsta-1,4,6-trien-3,17-dione	1.03	0.83	92
4	17β-Hydroxyandrosta-4,6-dien-3-one	0.75	0.73	80
5	17β-Hydroxyandrosta-1,4,6-trien-3-one	0.68	0.97	108
6	7α-Hydroxyandrosta-1,4-dien-3,17-dione	0.52	0.92	96
7	7α-Hydroxyandrost-4-en-3,17-dione	0.54	0.69	72
8	7α-Hydroxy-5β-cholan-3-oxo-24-oic acid	0.92	3.14	251
9	Chola-4,6-dien-3-one-24-oic acid	1.14	5.96	502
10	3-Oxo-4,6-pregnadien-20-carboxylic acid	1.12	0.33	30
11	7α-Hydroxy-3-oxo-1,4-pregnadien-20-carboxylic acid	0.44	0.72	63
12	7α-Hydroxy-3-oxo-4-pregnen-20-carboxylic acid	0.51	0.46	40

[a] R_f values for each compound calculated relative to ADD which was spotted near the side of preparative plates for reference.

are able to dehydroxylate cholic acid (20). Reports of 7α-dehydroxylation occurring under aerobic conditions, however, are few and in studies where this has occurred the explanations for the reactions differ. Severina et al. (21) isolated both C_{24} and C_{22} acidic 4,6-dienone steroids from an aerobically growing culture of *Mycobacterium* sp. on cholic acid. They suggested that the removal of the 7α-hydroxyl group was caused by facile dehydration during the separation methods used for isolating the compounds. Tenneson et al. isolated 1,4,6-trienone steroids from aerobic fermentations of CDCA (6) and cholic acid (22) by *Pseudomonas* sp. NCIB 10590 and suggested that the removal of the 7α-hydroxyl group was mediated by the bacterium.

The isolation of C_{24}, C_{22}, and C_{19} 4,6-dienone steroids in the present study indicates that removal of the 7α-hydroxyl group can occur at any stage during the anaerobic degradation of CDCA by the Pseudomonad. Compounds containing a single double bond at C_6–C_7 were not isolated and it seems probable that Δ^4-nuclear steroid dehydrogenation precedes removal of the 7α-hydroxyl group. 1,4,6-Trienone steroids were also isolated in rather lower yield, but were only of the C_{19} (androstane) type. This indicates that insertion of a double bond at C_1–C_2 in 4,6-dienone steroids can only occur after removal of the side-chain or else metabolism of 1,4,6-trienone acids is so rapid that they cannot be detected. 4,6-Dienone steroids are not produced from the aerobic metabolism of CDCA by the Pseudomonad (6) whilst they are present in large quantities under anaerobic conditions. 1,4,6-Trienone steroids, on the other hand, are produced under both aerobic and anaerobic conditions. This suggests that aerobiosis suppresses the induction of the 7α-dehydroxylating enzyme, whilst anaerobiosis is stimulatory.

In the absence of the 7α-dehydroxylating enzyme, the Pseudomonad would probably produce a higher percentage of 4-en-3-oxo steroids under anaerobic conditions because the ratio of 4-en-3-oxo steroids to 1,4-dienone steroids is higher under anaerobic than under aerobic conditions in the fermentation of deoxycholic acid (23) and lithocholic acid (24), both of which lack a 7α-hydroxyl group. It is suggested that the presence of oxygen stimulates the induction of a Δ^1-nuclear steroidal dehydrogenating enzyme and results in the dehydrogenation of 4,6-dienone steroids at C_1–C_2. This may explain the lack of 4,6-dienone steroids in the aerobic fermentation of CDCA.

The actual mechanism of 4,6-dienone production under anaerobic conditions can be explained thus. The lack of compounds possessing a double bond only at C_6–C_7 indicates that at least a 4-en-3-one steroid is the substrate for the 7α-dehydroxylase enzyme. 7α-Dehydroxylation of a 4-en-3-one steroid would result in the production of a double bond at C_6–C_7 which would become stabilized in conjunction with the 4-en-3-oxo group giving a 4,6-dien-3-oxo steroid (25).

It has been inferred that the production of 4,6-dienone steroids may be the result of facile dehydration of 7α-hydroxy-4-en-3-oxo steroids under mild acid conditions. However metabolites *7* and *12* remained stable under mild acid conditions (pH 4.0) for at least 3 weeks. In strong acid conditions (pH 1.0) at 55°C, 0.03 mg per ml solutions of *7* and *12* were completely converted to their 4,6-dienone derivatives only after 80 min incubation. It seems highly improbable that such reactions would occur during the extraction and separative procedures used in this study. This was corroborated because direct TLC of the culture broth also revealed 4,6-dienone steroids. Pure solutions of metabolites *7* and *12* were also stable in the TLC systems employed.

Although a significant accumulation of 4,6-dienone steroids occurred under anaerobic conditions, there was also substantial loss of steroidal material. It is probable

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that the androstane derivatives were degraded via aromatic secosteroids to nonsteroidal products in a manner similar to the degradation of androstanes produced by the Pseudomonad during the anaerobic metabolism of cholic and deoxycholic acids (26).

In conclusion, *Pseudomonas* sp. NCIB 10590 possesses a 7α-dehydroxylase enzyme that is induced by unsaturated 7α-hydroxy bile acid substrates under anaerobic conditions. A wide range of metabolites is produced and they are dominated by 4,6-dienone steroids.

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