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

Robert W. Owen, Michael J. Hill, and Rodney F. Bilton

Department of Chemistry and Biochemistry, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF England

**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 minor neutral products were androsta-4,6,14,17-tetraen-3,17-dione, 17a-hydroxyandrosta-4,6-dien-3,17-dione, 17α-hydroxyandrosta-1,4-dien-3,17-dione, 7α-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-hydroxyandrosta-1,4-dien-3,17-dione. In contrast to aerobic catabolism of CDCA with *E. coli* grown under anaerobic 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 *P. NCIB* 10590 in anaerobic conditions.

**MATERIALS**

CDCA, 5α-cholestanate, 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 spectrometer.

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-4,6-dien-3,17-dione.

1 To whom reprint requests should be sent.

2 Present address: Public Health Laboratory Service, Centre for Applied Microbiology and Research, Bacterial Metabolism Research Laboratory, Porton Down, Salisbury, Wiltshire SP4 0JG, England.
spectrum 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 Supel-
coport 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-cholestan with a flow rate of 30 ml min⁻¹ nitrogen
through the column and 30 ml min⁻¹ H₂ to the flame
ionization detector in a Hewlett-Packard H.P. 5470 in-
strument.

Thin-layer chromatography (TLC)
Analysis by TLC was performed on 0.25-mm layers
of Kieselgel GF₃₄₄ (obtained from E. Merck, Darmstadt,
West Germany) in methanol–dichloromethane 1:19 (v/v)
and the mobilities were measured relative to an-
drosta-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 ultra-
violet 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 (1
0.0025; ZnSO₄·H₂O, 0.0025; and MnSO₄·4H₂O,
0.3; K₂HPO₄, 0.7; KH₂PO₄,
0.3; KNO₃, 1.0; MgSO₄·7H₂O, 0.1; FeSO₄·7H₂O, 0.0025; 
ZnSO₄·7H₂O, 0.0025; and MnSO₄·4H₂O, 0.0025; final pH 7.2. Solutions of sodium chenodeoxy-
cholate, mineral salts, trace elements, and magnesium
sulfate were autoclaved separately prior to admixture.
Shake-flask cultures were grown on an L. H. Engine-
rating 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 × 10⁶ cells ml⁻¹ (OD of 1.0 at 540
nm). The culture medium was steamed for 1 hr im-
mediately before inoculation to remove dissolved oxy-
gen. The top of the fermentation bottle was loosened
(to allow gaseous exchange) prior to incubation at 28°C
for 6 weeks under 90% H₂–10% CO₂ (with palladium
catalyst to remove residual oxygen). The course of the
fermentation and OD₅₄₀ was followed at weekly inter-
vals. After 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 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–meth-
anol 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 sepa-
rated likewise giving steroids 2–7.

Androsta-4,6-dien-3,17-dione (2)
Recrystallization of 2 (Fig. 1) from methanol–dicho-
rromethane gave yellow needles (152 mg); mp 172–
173°C; IR, 1746 (17-ketone), 1664 (3-ketone), 1616
and 1582 cm⁻¹ (C₄–C₅ and C₆–C₇ double bonds); UV,
284 nm (ε 28,350); NMR, (δ) 0.97, 1.15 (6H, s, 18-CH₃
and 19-CH₃); 2.35–2.65 (2H, m, 16-CH₂), 6.06–6.37 (4H, 
m, 2-H, 4-H, 5-H, and 7-H); MS, M⁺ 284 (74%, C₁₉H₂₄O₂ requires M⁺, 284), m/e 136 (4,6-diene-3-one,
100%); GLC, Rf 1.50; TLC, Rf 1.08; after oxidation,
Rf 1.08; after acetylation, Rf 1.08; and after reduction
Rf 0.75.

Androsta-1,4,6-trien-3,17-dione (3)
Recrystallization of 3 (Fig. 1) from methanol–dicho-
rromethane gave white crystals (92 mg); mp 172–
173°C; IR, 1746 (17-ketone), 1664 (3-ketone), 1616
and 1582 cm⁻¹ (C₄–C₅ and C₆–C₇ double bonds); UV,
224, 256, and 300 nm (ε 12,740, 10,500, and 13,670);
NMR, (δ) 1.00, 1.23 (6H, s, 18-CH₃ and 19-
CH₃), 2.40–2.65 (2H, M, 16-CH₂), 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%, C₁₉H₂₂O₂ requires M⁺, 282), m/e 
134 (1,4,6-trien-3-one, 100%); GLC, Rf 1.60; TLC,
Rf 1.03; after oxidation, Rf 1.03; after acetylation, Rf
1.03; and after reduction, Rf 0.68.

17β-Hydroxyandrosta-4,6-dien-3-one (4)
Recrystallization of 4 (Fig. 1) from methanol–dicho-
rromethane gave yellow spiky needles (80 mg); mp 198–
200°C; IR, 3405 (hydroxyl), 1656 (3-ketone), 1620 and
1586 cm⁻¹ (C₄–C₅ and C₆–C₇ double bonds); UV, 285
nm (ε 22,017); NMR, (δ) 0.83, 1.12 (6H, s, 18-CH₃ and
OH 9H

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

17α-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, 3345 (hydroxyl), 1661 (17-ketone), 1660 (3-ketone), and 1610 cm⁻¹ (C₄–C₅ double bond); UV, 244, 256, 300 nm (λ 13,000, 12,250, and 14,980); NMR, (δ) 0.95, 1.14 (6H, s, 18-CH₃ and 19-CH₃), 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%, C₁₉H₂₆O₂ 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, H₂O, 32%); GLC, Rf 1.50; TLC, Rf 0.68; after oxidation, Rf 1.03; after acetylation, Rf 1.09; and after reduction, Rf 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), 1660 (3-ketone), 1610 and 1596 (C₁–C₂ and C₄–C₅ double bonds); UV, 244 nm (λ 16,500); NMR, (δ) 0.95, 1.26 (6H, s, 18-CH₃ and 19-CH₃), 2.40–2.58 (2H, m, 16-d, showing further splitting, J = 10 Hz, 2-H), 7.07 (1H, d, J = 10 Hz, 1-H); MS, M⁺ 300 (19%, C₁₉H₂₄O₃ requires M⁺ 300), base peak at m/e 122 (1,4-dien-3-one, 100%) and low intensity ion at m/e 282 (M⁺–18, H₂O, 22%); GLC, Rf 2.50; TLC, Rf 0.54; after oxidation, Rf 1.03; after acetylation, Rf 1.05; and after reduction, Rf 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 powdery crystals (72 mg); mp 225–226°C; IR, 3340 (hydroxyl), 1740 (17-ketone), 1660 (3-ketone), and 1610 cm⁻¹ (C₄–C₅ double bond); UV, 244 nm (λ 13,000); NMR, (δ) 0.70, 1.10 (6H, s, 18-CH₃ and 19-CH₃), 0.94 (3H, s, 21-CH₃), 3.68 (3H, s, 24-OCH₃) and 5.78 (1H, t, J = 3 Hz, 7-H); MS, M⁺ 404 (26%, C₂₅H₄₀O₄ requires M⁺ 404), low intensity ion at m/e 386 (M⁺–18, H₂O, 40%) and base peak at m/e 271 (M⁺–133 6C-side chain + H₂O, 100%); GLC, Rf 9.30; TLC, Rf 0.92; after oxidation, Rf 1.13; after acetylation, Rf 1.10; and after reduction, Rf 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 (C₄–C₅ and C₆–C₇ double bonds); UV, 284 nm (λ
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-CH₃ and 19-CH₃), 0.93 (3H, d, J = 5 Hz, 21-CH₃), 3.68 (3H, s, 24-OCH₃), 5.67 (1H, s-4-H); MS, M⁺ 384 (100%, C₂₅H₃₆O₃ 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, Rₜ 7.00; TLC, Rₜ 1.14; after oxidation, Rₜ 1.14; after acetylation, Rₜ 1.14; and after reduction, Rₜ 1.14.

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

Recrystallization of methyl 10 (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 (C₄-C₅ double bonds); UV, 242 nm (ε 14,600); MS, M⁺ 374 (31%, C₂₃H₃₂O₄ requires M⁺ 374), base peak at m/e 124 (4-en-3-one, 100%), intense ion at m/e 356 (M⁺-18, H₂O, 94%) and low intensity ion at m/e 269 (M⁺-105, 4C-side chain + H₂O, 33%); GLC, Rₜ 6.60; TLC, Rₜ 0.51; after oxidation, Rₜ 1.19; after acetylation, Rₜ 1.21; and after reduction, Rₜ 0.51.

Methyl 7α-hydroxy-3-oxo-1,4-pregnadien-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 (C₄-C₅ double bonds); UV, 242 nm (ε 14,600); MS, M⁺ 374 (31%, C₂₃H₃₂O₄ requires M⁺ 374), base peak at m/e 124 (4-en-3-one, 100%), intense ion at m/e 356 (M⁺-18, H₂O, 94%) and low intensity ion at m/e 269 (M⁺-105, 4C-side chain + H₂O, 33%); GLC, Rₜ 6.60; TLC, Rₜ 0.51; after oxidation, Rₜ 1.19; after acetylation, Rₜ 1.21; and after reduction, Rₜ 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 (λmax, 284 nm, di-β-substituted a,β-unsaturated ketone in a six-membered ring, double bond exocyclic, extended by a double bond at C6-C7) (12), IR spectra (1664, 1616, 1582 and 1656, 1620, 1586 cm⁻¹, aβ-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 RₜADD 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-
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 $\delta$ which is characteristic for a $17\alpha$-proton (13). The $17\alpha$-proton presumably bisects the dihedral angle between the two protons at C16 with a coupling constant of 8 Hz. The hydroxyl group in 4 was thus assigned the $17\beta$-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 $\beta$-equatorial hydroxyl groups than with $\alpha$-axial hydroxyl groups. As a result the ion due to the loss of $\beta$-hydroxyl groups is less intense than the molecular ion, whilst the ion due to the loss of $\alpha$-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 intensi-

![Mass Spectrum of Androsta-4,6-dien-3,17-dione](image)

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

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 ($\lambda_{max}$, 224, 256, 300 nm, di-$\beta$-substituted, $\alpha,\beta$-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$^{-1}$, respec-

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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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%).
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 1619 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 C7 hydroxyl functions are subject to dehydration under the conditions used, the hydroxyl group in 7 was deemed to be at C7. 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 C7 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
the loss of the full bile acid side chain (i.e., 6C-side chain + H2O). The PMR spectrum did not reveal any vinylic protons; however a triplet was present at 5.78 \( \delta \) typical of a 7\( \beta \)-proton on a carbon carrying a 7\( \alpha \)-hydroxyl group. The mass and PMR spectra indicated that the hydroxyl group in methyl 8 was of the 7\( \alpha \)-axial configuration. Metabolite 8 was therefore assigned to the structure 7\( \alpha \)-hydroxy-5\( \beta \)-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 (\( \lambda_{max} \), 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 \( \delta \) 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 7\( \alpha \)-hydroxy-3-oxo-1,4-pregnadien-20-carboxylic acid and 7\( \alpha \)-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 7\( \alpha \)-hydroxy-1,4-dienone steroids as the major intermediates (6). The removal of the hydroxyl group at C7 suggests that induction of a 7\( \alpha \)-dehydroxylase enzyme occurs under anaerobic conditions.

It has been shown that the induction of 7\( \alpha \)-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 7\( \alpha \)-dehydroxylation of cholic acid is a diaxial trans elimination of the 7\( \alpha \)-hydroxyl group and the 6\( \beta \)-hydrogen atom yielding 3\( \alpha \),12\( \alpha \)-dihydroxy-5\( \beta \)-chol-6-en-24-oic acid. In the human intestine, where 7\( \alpha \)-dehydroxylation of bile acids is very common (19), it has been shown that the \( \Delta^6 \)-acid is then reduced by trans hydrogenation at the 6\( \alpha \)- and 7\( \beta \)-positions to give deoxycholic acid (19).

7\( \alpha \)-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,*
aerobiosis is stimulatory.

Compounds containing a single double bond at C6-C7 were not isolated and it seems probable that A4-nuclear steroid dehydrogenation precedes removal of the 7a-hydroxyl group. Reports of aerobic metabolism of CDCA by the Pseudomonad (6) and cholic acid (22) by Mycobacterium sp. on cholic acid. They suggested that the removal of the 7a-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 7a-hydroxy group was mediated by the bacterium. The isolation of C24, C22, and C19 4,6-dienone steroids in the present study indicates that removal of the 7a-hydroxyl group can occur at any stage during the anaerobic degradation of CDCA by the Pseudomonad. Compounds containing a single double bond at C6-C7 were not isolated and it seems probable that A4-nuclear steroid dehydrogenation precedes removal of the 7a-hydroxy group. 1,4,6-Triene steroids were also isolated in rather lower yield, but were only of the C19 (androstanane) type. This indicates that insertion of a double bond at C1-C2 in 4,6-dienone steroids can only occur after removal of the side-chain or else metabolism of 1,4,6-dienone acids is so rapid that they cannot be detected. 4,6-Diene 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-Triene steroids, on the other hand, are produced under both aerobic and anaerobic conditions. This suggests that aerobic metabolism of CDCA by the Pseudomonad (6) anaerobic degradation of CDCA by the Pseudomonad. 1,4,6-Triene steroids may be the result of facile dehydration of 7a-hydroxy-4-en-3-oxo steroids under anaerobic 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-diene 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-diene steroids. Pure solutions of metabolites 7 and 12 were also stable in the TLC systems employed. Although a significant accumulation of 4,6-diene steroids occurred under anaerobic conditions, there was also substantial loss of steroidal material. It is probable

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

<table>
<thead>
<tr>
<th>Metabolite Number</th>
<th>Metabolite</th>
<th>Yield (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Androsta-4,6-dien-3,17-dione</td>
<td>1.37</td>
<td>152</td>
</tr>
<tr>
<td>3</td>
<td>Androsta-4,6-dien-3,17-dione</td>
<td>0.83</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>17β-Hydroxyandrost-4,6-dien-3-one</td>
<td>0.75</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>17β-Hydroxyandrost-4,6-dien-3-one</td>
<td>0.97</td>
<td>108</td>
</tr>
<tr>
<td>6</td>
<td>7α-Hydroxyandrost-4,6-dien-3,17-dione</td>
<td>0.92</td>
<td>96</td>
</tr>
<tr>
<td>7</td>
<td>7α-Hydroxyandrost-4,6-dien-3,17-dione</td>
<td>0.69</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>7α-Hydroxy-5β-cholan-3-oxo-24-oic acid</td>
<td>1.14</td>
<td>502</td>
</tr>
<tr>
<td>9</td>
<td>Chola-4,6-dien-3,17-dione</td>
<td>5.96</td>
<td>300</td>
</tr>
<tr>
<td>10</td>
<td>5-Oxo-4,6-pregnadien-20-carboxylic acid</td>
<td>0.53</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>7α-Hydroxy-5-oxo-1,4-pregnadien-20-carboxylic acid</td>
<td>0.72</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>7α-Hydroxy-5-oxo-4-pregnadien-20-carboxylic acid</td>
<td>0.46</td>
<td>40</td>
</tr>
</tbody>
</table>

* a Y values for each compound calculated relative to ADD which was spotted near the side of preparative plates for reference.
that the androstan derivatives were degraded via aromatic secosteroids to nonsteroidal products in a manner similar to the degradation of androstanes produced by the Pseudomonas during the anaerobic metabolism of cholic and deoxycholic acids (26).

In conclusion, Pseudomonas sp. NCIB 10590 possesses a 7α-dehydroxylation 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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REFERENCES