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THE DEGRADATION OF HYODEOXYCHOLIC ACID BY *PSEUDOMONAS* Spp. N.C.I.B. 10590

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SUMMARY

The microbial degradation of hyodeoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590 has been studied. Two major products and one minor product have been isolated and identified as *6α-hydroxyandrosta-1,4-dien-3,17-dione*, *6α-hydroxy-3-oxopregna-1,4-dien-20-carboxylic acid* and *androsta-1,4-dien-3,6,17-trione* respectively. Two other minor products were isolated and evidence is given for the following structures: *6α-hydroxyandrost-4-en-3,17-dione* and *6α-hydroxy-3-oxopregn-4-en-20-carboxylic acid*. A possible paathway of hyodeoxycholic acid degradation is suggested.

INTRODUCTION

The production of physiologically active steroids from the microbial degradation of bile acids [1] is of potential importance commercially. The microbial degradation of the more common bile acids has been studied [2, 3] but there is little evidence of any investigation into the microbial degradation of hyodeoxycholic acid. The only reported observation of this kind involves a strain of *Escherichia coli* isolated from a faecal sample of a colon cancer patient. This isolate has the ability to degrade hyodeoxycholic acid under anaerobic conditions [4].

The degradation of bile acids by bacteria present in the human gut has been implicated in the aetiology of colon [5] and breast [6] cancer. Although hyodeoxycholic acid is not a naturally occurring bile acid in humans it may be fed to patients suffering from gallstones [7]. This would mean relatively large amounts of hyodeoxycholic acid would be present in the gut of such patients, enabling microbial degradation to take place.

In this paper we present evidence for the structure and configuration of the two main (2 and 5) and one minor (3) side-chain cleavage products isolated during the aerobic degradation of hyodeoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590. Two other minor compounds have been isolated and their structures are discussed. The isolation of these metabolites has enabled the postulation of a pathway of hyodeoxycholic acid degradation.

EXPERIMENTAL

Hyodeoxycholic acid, 5α-cholestane and androsta-1,4-dien-3,17-dione were obtained from Koch Light. General reagents were of Analar grade and obtained from B.D.H. and all solvents were redistilled before use.

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. Elemental analyses were determined by the Butterworth Microanalytical Consultancy. Infra red spectra were determined from KBr discs on a Perkin-Elmer 457 spectrophotometer. Ultraviolet spectra were determined for solutions in methanol on a Pye-Unicam SP 1800 spectrophotometer. Nuclear magnetic resonance proton spectra were recorded on a Varian HA 100 spectrometer from solutions in deuterated chloroform. Mass spectra were recorded on an A.E.I. MS 12 spectrometer.

Analysis by gas chromatography was performed at 260° using 3% OV-17 on 80/100 mesh "Supelcoport" in a 1.5 m × 3 mm column obtained from Phase Separations. Retention times were measured relative to 5α-cholestane with a flow rate of 30 ml min^{-1} nitrogen in a Hewlett-Packard HP 5470 instrument. Analysis by thin layer chromatography was performed on 0.25 mm layers of Kieselgel GF_{254}, obtained from Merck, in methanol–dichloromethane (1:9, V/V) and the mobilities were measured relative to androsta-1,4-dien-3,17-dione. Products containing a 4-en-3-one or a 1,4-dien-3-one structure were detected under ultraviolet light; other products were detected by their colour with anisaldehyde reagent [8]. Isonicotinic acid hydrazide(INH) spray reagent [9] was used to distinguish between 4-en-3-oxo and 1,4-dien-3-oxo steroids. Purification was achieved by preparative thin layer chromatography on Kieselgel GF_{254} as above.

Oxidation was performed on thin layer chromatography by overspotting with a solution of Jones' chromic reagent [10] diluted 1:4 with acetone. Ace-

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tylation was performed by overspotting with acetyl chloride. Reduction was performed by overspotting with potassium borohydride reagent (1 g KBH_4, 1 ml 2N NaOH, 8 ml H_2O). Acidic steroids were methylated with diazomethane. Trimethylsilyl (TMS) ethers were prepared using bis-trimethylsilyl acetamide.

The culture medium consisted of sodium hyodeoxycholate (1.0 g); K_2HPO_4 (1.6 g), KH_2PO_4 (0.4 g), KNO_3 (1.0 g) (mineral salts); $FeSO_4 \cdot 7H_2O$ (2.5 mg), $ZnSO_4 \cdot 7H_2O$ (2.5 mg), $MnSO_4 \cdot 4H_2O$ (2.5 mg) (trace elements); $MgSO_4 \cdot 7H_2O$ (0.1 g) and distilled water to one litre (pH 7.2). Solutions of sodium hyodeoxycholate, mineral salts, trace elements and magnesium sulphate were autoclaved separately before mixing. Shake-flask cultures were grown on an L.H. Engineering orbital incubator and the ten litre culture was grown in an L.H. Engineering Fermenter with constant aeration of 2 l/min and stirring at 200 rev/min. The incubation temperature was 28°C at all times.

The cells obtained from a one litre shake-flask culture of *Pseudomonas* spp. N.C.I.B. 10590 by centrifugation at 10,000 *g* on a MSE Mistral 4L centrifuge were used to inoculate ten litres of the culture medium. The course of the transformation was followed by sampling the culture at one hour intervals. The cell density was calculated from the absorbance of the culture at 540 nm. Filtration of the samples through a 0.45 μm Millipore filter enabled the direct determination of the absorbance at 252 nm of the steroidal mixture. Extraction of the samples with ethyl acetate followed by thin layer chromatography and gas chromatography analysis enabled determinations of the relative concentrations of hyodeoxycholic acid, the major acidic and the major neutral steroids. When the absorbance at 252 nm reached a maximum, after 18 h, the culture was terminated by direct extraction of the metabolites into dichloromethane (4 × 1 l.). After drying over $MgSO_4$ the solvent was removed under reduced pressure at 30°C to yield 2.3 g of a tarry residue. The residue was then taken up in warm dichloromethane (10 ml) and the mixture was separated by preparative thin layer chromatography into a series of fractions from which steroids **2, 3, 4, 5** and **6** were crystallized.

6α-hydroxyandrosta-1,4-dien-3,17-dione (**2**). Recrystallization of **2** from methanol/dichloromethane yielded white prisms (16 mg) m.p. 240–241°C. (Found: C, 75.69; H, 7.97. $C_{19}H_{24}O_3$ requires: C, 76.00, H, 8.00%). ν_{max} 3480 (hydroxyl), 1737 (17-ketone), 1654 (3-ketone), 1618 and 1603 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); λ_{max} 244 nm (ϵ 14,470); δ 0.97, 1.26 (6H, s, 18-CH_3 and 19-CH_3), 2.30–2.50 (2H, m, 16-CH_2), 2.94 (1H, s, 6-OH), 4.55 (1H, m, 6β-H), 6.30 (1H, d, showing further splitting, J = 10 Hz, 2-H), 6.56 (1H, s, slight splitting, 4-H) and 7.08 (1H, *d*, J = 10 Hz, 1-H); M^+ 300 ($C_{19}H_{24}O_3$ requires M^+ 300), *m/e* 138 (1,4-dien-3-one-6-ol) and *m/e* 282 (M^+-H_2O). GLC (TMS ether of **2**) R_F 1.8; TLC R_F 0.77, after oxidation R_F 1.01, after acetylation R_F 1.02 and after reduction R_F 0.48.

Androsta-1,4-dien-3,6,17-trione (**3**). Recrystallization of **3** from methanol/dichloromethane yielded pale yellow needles (3 mg) m.p. 211–212°C. (Found: C, 76.46; H, 7.20. $C_{19}H_{22}O_3$ requires: C, 76.51; H 7.38%). ν_{max} 1720 (17-ketone), 1680 (6-ketone), 1647 (3-ketone), 1618 and 1597 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); λ_{max} 250 nm (ϵ 13,000); δ 0.98, 1.25 (6H, s, 18-CH_3 and 19-CH_3), 6.30 (1H, *d*, showing further splitting J = 10 Hz, 2-H), 6.38 (1H, s, slight splitting, 4-H) and 7.08 (1H, *d*, J = 10 Hz, 1-H); M^+ 298 ($C_{19}H_{22}O_3$ requires M^+ 298) and *m/e* 135 (1,4-dien-3,6-dione). GLC R_F 2.3; TLC R_F 1.01, after oxidation R_F 1.01, after acetylation R_F 1.01 and after reduction R_F 0.52.

6α-hydroxandrost-4-en-3,17-dione (**4**). Present as an impure solid (1 mg). λ_{max} 240 nm; mass spectrum (TMS ether of **4**) M^+ 374 ($C_{22}H_{34}O_3Si$ requires M^+ 374), *m/e* 212 (4-en-3-one-6-TMS ether) and *m/e* 284 (M^+-H_2O)·GLC (TMS ether of 4) R_F 1.5; TLC R_F 0.80, after oxidation R_F 1.06, after acetylation R_F 1.04 and after reduction R_F 0.50.

Methyl 6α-hydroxy-3-oxopregna-1,4-dien-20-oate (methyl ester of **5**). Recrystallization of methyl **5** from methanol/dichloromethane yielded white prisms (9 mg) m.p. 253–254°C. (Found: C, 73.99; H, 8.73. $C_{23}H_{32}O_4$ requires: C, 74.20; H, 8.60%). ν_{max} 3492 (hydroxyl), 1718 (carboxyl), 1654 (3-ketone), 1620 and 1602 cm^{-1} (C_1-C_2 and C_4-C_5 double bonds); λ_{max} 244 nm (ϵ 14,510); δ 0.80, 1.24 (6H, s, 18-CH_3 and 19-CH_3), 1.26 (3H, *d*, J = 6 Hz, 21-CH_3), 3.71 (3H, s, 22-OCH_3), 4.52 (1H, m, 6β-H), 6.29 (1H, *d*, showing further splitting, J = 10 Hz, 2-H), 6.55 (1H, s, slight splitting, 4-H) and 7.06 (1H, *d*, J = 10 Hz, 1-H); M^+ 372 ($C_{23}H_{32}O_4$ requires M^+ 372) *m/e* 138 (1,4- dien-3-one-6-ol), *m/e* 267 (M^+-side-chain + H_2O) and *m/e* 354 ($M^+ - H_2O$). GLC (TMS ether of methyl 5) R_F 4.3; TLC R_F 0.78; after oxidation R_F 1.02, after acetylation R_F 1.07 and after reduction R_F 0.78.

Methyl 6α-hydroxy-3-oxopregn-4-en-20-oate (methyl ester of **6**). Recrystallisation of methyl **6** from methanol/dichloromethane yielded white prisms (10 mg) m.p. 246–247°C. ν_{max} 3472 (hydroxyl), 1722 (carboxyl), 1655 (3-ketone) and 1618 (C_4-C_5 double bond); λ_{max} 241 nm (ϵ 15,640); δ 0.82, 1.23 (6H, s, 18-CH_3 and 19-CH_3), 1.25 (3H, *d*, J = 6 Hz, 21-CH_3), 3.72 (3H, s, 22-OCH_3), 4.54 (1H, m, 6β-H) and 6.45 (1H, s, 4-H); M^+ 374 ($C_{23}H_{34}O_4$ requires M^+ 374), *m/e* 140 (4-en-3-one-6-ol), *m/e* 269 (M^+-side-chain + H_2O) and *m/e* 356 (M^+-H_2O). GLC (TMS ether of methyl **6**) R_F 3.6; TLC R_F 0.84, after oxidation R_F 1.06, after acetylation R_F 1.10 and after reduction R_F 0.84.

Phenolic compounds. A crude mixture of compounds remained after the removal of the steroidal metabolites. λ_{max} 218 and 275 nm (methanol), 220 and 298 nm (NaOH methanol). GLC Two main metabolites R_F 0.2 and 0.8; TLC Two main metabolites R_F 0.79 and 0.70, other metabolites R_F 0.91 and 0.40.

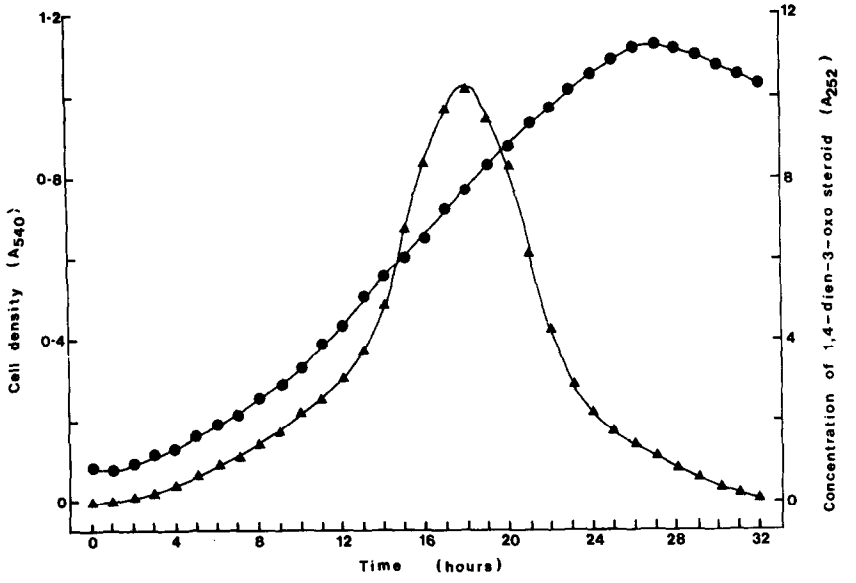


Fig. 1. The relationship between cell density (●——●) and concentration of 1,4-dien-3-oxo steroid (▲——▲) during the oxidation of hyodeoxycholic acid (1) by *Pseudomonas* spp. N.C.I.B. 10590.

RESULTS

Pseudomonas spp. N.C.I.B. 10590 grew well on sodium hyodeoxycholate, in a mineral salts medium. The course of the transformation was followed by measurement of the increase in both cell density and the concentration of 1,4-dien-3-oxo steroids (Fig. 1). The growth of the *Pseudomonas* species on sodium hyodeoxycholate showed a typical lag, log and stationary phase, while the concentration of 1,4-dien-3-oxo steroids in the medium showed a maximum after 18 h. The concentration of hyodeoxycholic acid was shown to decrease with time, whereas the concentration of the major acidic and neutral, 1,4-dien-3-oxo steroids reached a maximum after 16 h and 19 h respectively (Fig. 2).

The metabolites isolated after 18 h transformation of hyodeoxycholic acid (1) are shown in Fig. 3. The major neutral compound (2) was isolated as a crystalline solid, the mass spectrum of which shows a molecular ion at *m/e* 300. The infra red spectrum (1654, 1618 and 1603 cm^{-1}, $\alpha\beta$-unsaturated ketone), the ultraviolet spectrum (λ_{max} 244 nm, di-β-substituted $\alpha\beta$-unsaturated ketone in a six-membered ring, double bond exocyclic) [11] and the nuclear magnetic resonance proton spectrum (three vinylic protons in the range 6.30–7.08δ) of 2 suggest a steroidal

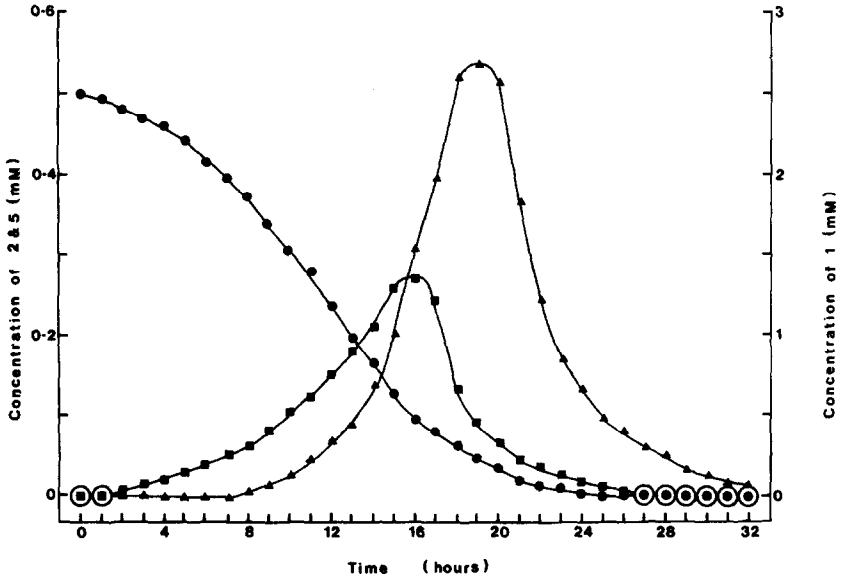


Fig. 2. The relationship between the concentration of hyodeoxycholic acid (1) (●——●), the major neutral (2) (▲——▲) and acidic (5) (■——■) 1,4-dien-3-oxo steroids during the degradation of hyodeoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590.

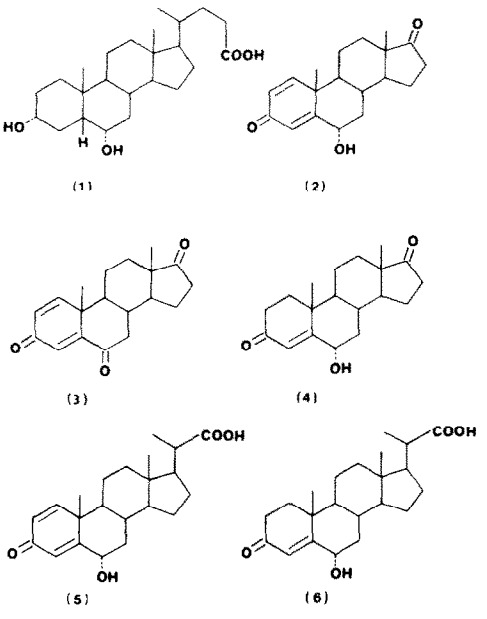


Fig. 3. Metabolites isolated from the degradation of hyodeoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590.

1,4-dien-3-one A ring structure. An intense ion at *m/e* 138 in the mass spectrum of **2** suggests the presence of a steroidal 1,4-dien-3-one-6-ol structure [12]. Compound **2** is easily oxidized, acetylated and reduced suggesting the presence of both a hydroxyl and a ketone group. This is confirmed by the infra red spectrum which contains a peak at 3480 cm^{-1} characteristic of hydroxyl groups and a peak at 1737 cm^{-1} characteristic of a ketone group in a five-membered ring. The nuclear magnetic resonance proton spectrum of **2** shows a broad multiplet centred at 4.55δ. The dihedral (Karplus) angles for the 6β proton and the two protons at C_7 are about 60° and 180° giving two different coupling constants. A four-line multiplet would, therefore, be expected, but the pattern is probably further complicated by allylic coupling to the proton at C_4. On this basis the hydroxyl group of **2** is assigned the 6α configuration [13]. 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 more readily with axial hydroxyl groups than with equatorial hydroxyl groups. With compound **2** a low intensity ion was observed at *m/e* 282 in the mass spectrum indicating the presence of a 6α equatorial hydroxyl group. Compound **2** is therefore assigned the structure *6α-hydroxyandrosta-1,4-dien-3,17-dione.*

Two minor neutral compounds (**3, 4**) were isolated and the mass spectrum of **3** shows a molecular ion at *m/e* 298 and an intense ion at *m/e* 135 suggesting a steroidal 1,4-dien-3,6 dione structure [12]. Confirmation of this structure is provided by the ultraviolet spectrum (λ_{max} 250 nm, di-β-substituted α,β-unsaturated ketone in a six-membered ring, double bond exocyclic, extended by a carbonyl group at C_6) [11]. The infra red and the nuclear magnetic resonance proton spectra of compound **3** provide confirmation of the 1,4-dien-3-one A ring structure as before. Compound **3** resists both oxidation and acetylation corresponding to the oxidation product of **2**. Compound **3** is easily reduced suggesting the presence of a ketone group. This is confirmed by the infra red spectrum which contains a peak at 1680 cm^{-1} characteristic of a ketone group and a peak at 1720 cm^{-1} characteristic of a ketone group in a five-membered ring. Compound **3** is therefore assigned the structure *androsta-1,4-dien-3,6,17-trione.*

The trimethylsilylether (TMS-ether) of **4** shows a molecular ion at *m/e* 374 and an intense ion at *m/e* 212 in the mass spectrum, suggesting a steroidal 4-en-3-one-6-TMS-ether structure [12]. The ultraviolet spectrum of **4** (λ_{max} 240 nm, di-β-substituted α,β-unsaturated ketone in a six-membered ring, double bond exocyclic) [11] and the immediate development of an intense yellow coloration with isonicotinic acid hydrazide spray (INH) [9], provide some confirmation of the 4-en-3-one A ring structure. Compound **4** is easily oxidized, acetylated and reduced suggesting the presence of both a hydroxyl and a ketone group. Thin layer chromatographic analysis shows **4** is slightly less polar than **2** and the oxidation product of **4** is slightly less polar than **3**. This shows that the oxygen substitution pattern is probably the same in all three compounds, the slight difference in polarity being caused by the different A ring structures. Insufficient pure **4** is available to obtain nuclear magnetic resonance proton spectra so the stereo-chemistry of the hydroxyl group is inferred from the mass spectral analysis. The TMS-ether of **4** shows a low intensity ion at *m/e* 284 in the mass spectrum indicating the presence of a 6α equatorial TMS-ether [14]. Compound **4** is, therefore, tentatively assigned the structure *6α-hydroxyandrost-4-en-3,17-dione.*

The major acidic steroid (**5**) was isolated as a crystalline solid the methyl ester of which shows a molecular ion at *m/e* 372. The infra red, the ultraviolet and the nuclear magnetic resonance proton spectrum of methyl **5** suggest a steroidal 1,4-dien-3-one A ring structure as before. An intense ion at *m/e* 138 in the mass spectrum of methyl **5** suggests the presence of a steroidal 1,4-dien-3-one-6-ol structure. Compound **5** is easily oxidized and acetylated but resists reduction suggesting the presence of a hydroxyl group. This is confirmed by the infra red spectrum. The nuclear magnetic resonance proton spectrum of methyl **5** shows a broad multiplet centred at 4.52 δ and on this basis the hydroxyl group of **5** is assigned the 6α configuration. Confirmation for this assignment is provided by the presence of a low intensity ion at *m/e* 354 in the mass spectrum. An intense ion at *m/e* 267 in the mass spectrum of methyl **5** corresponds to loss of the side-chain from C_{17}. Compound **5** is therefore assigned the structure *6α-hydroxy-3-oxopregna-1,4-dien-20-carboxylic acid.*

Another acidic compound (**6**) was isolated, the methyl ester of which shows a molecular ion at *m/e* 374. The infra red spectrum (1655 and 1618 cm^{-1}, $\alpha\beta$-unsaturated ketone), the ultraviolet spectrum (λ_{max} 241 nm, di-β-substituted $\alpha\beta$-unsaturated ketone in a six-membered ring, double bond exocyclic) [11] and the nuclear magnetic resonance proton spectrum (one vinylic proton at 6.45δ) of methyl **6** suggest a steroidal 4-en-3-one A ring structure. An intense ion at *m/e* 140 in the mass spectrum of methyl **5** suggests the presence of a steroidal 4-en-3-one-6-ol structure [12]. Compound **6** is easily oxidized and acetylated but resists reduction suggesting the presence of a hydroxyl group. This is confirmed by the infra red spectrum. The hydroxyl group is assigned the 6α configuration from the nuclear magnetic resonance proton spectrum. Again a broad multiplet centred at 4.54δ is shown. A low intensity ion at *m/e* 356 and an intense ion at *m/e* 269 in the mass spectrum, confirmed the loss of a 6α hydroxyl group and the side-chain from C_{17}. Compound **6** is therefore assigned the structure *6α-hydroxy-3-oxopregn-4-en-20-carboxylic acid.*

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

A crude mixture of compounds was left after the removal of the steroidal metabolites. This mixture contained two main metabolites, both of which gave very short retention times on gas chromatography suggesting that they are low molecular weight compounds. The ultraviolet spectrum of the mixture (λ_{max} 218 and 275 nm) which showed a bathochromic shift in alkaline solution (λ_{max} 220 and 298 nm) indicated the presence of phenolic compounds.

DISCUSSION

The isolation and identification of steroidal metabolites **2, 3, 4, 5** and **6** during the degradation of hyodeoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590 is the first recorded instance of the microbial degradation of hyodeoxycholic acid under aerobic conditions. Hyodeoxycholic acid is freely available in pig bile and could be used to produce physiologically active steroids by microbial degradation and subsequent chemical modification.

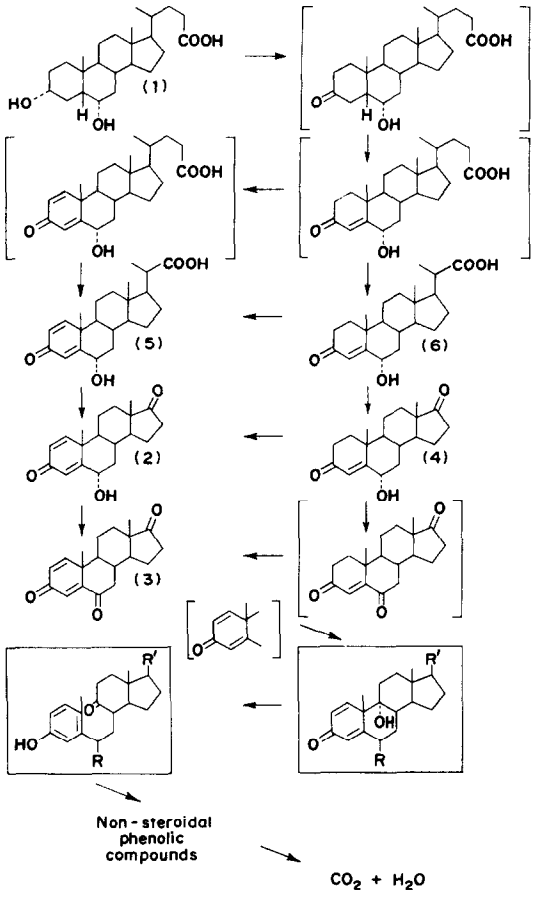
The microbial degradation of bile acids has been studied for many years but as yet no one pathway has been established to occur universally. A pathway for both lithocholic acid [15] and chenodeoxycholic acid [16] degradation has been postulated and the metabolites isolated during the degradation of hyodeoxycholic acid follow a similar pattern. All products containing a double bond in the A ring possess a ketone group at C_3 [2, 3, 15, 16] and it is therefore suggested that 3α-hydroxysteroid dehydrogenation precedes the dehydrogenation of ring A. Dehydrogenation between C_1 and C_2 presumably occurs after dehydrogenation between C_4 and C_5, since no products containing a single double bond at C_1-C_2 have been isolated [2, 3, 15, 16]. The products isolated and identified from the degradation of deoxycholic acid by *Pseudomonas* spp. N.C.I.B. 10590 exhibit a ratio of 4-en-3-oxo to 1,4-dien-3-oxo steroids from 1:1 (C_{24}), 1:4 (C_{22}) to 1:25 (C_{19}) [17], suggesting that dehydrogenation between C_1 and C_2 may occur at any stage during bile acid degradation. It is not known whether or not 6α-hydroxysteroid dehydrogenation can occur at any stage during bile acid degradation since compound **3** is the first reported 6-oxo metabolite.

It has been postulated [2] that for the cleavage of the bile acid side-chain to occur it must first be transformed to at least a 4-en-3-oxo steroid. This hypothesis is supported by the fact that all side-chain cleavage products isolated so far are such metabolites. The mechanism of side-chain cleavage probably occurs by β-oxidation from a C_{24} bile acid through a C_{22} metabolite to a C_{19} androstane. This is illustrated in the degradation of hyodeoxycholic acid (**1**) (C_{24}) by *Pseudomonas* spp. N.C.I.B. 10590 where compound **5** (C_{22}) is produced in the medium before compound **2** (C_{19}) (Fig. 2).

Cholic acid and lithocholic acid have been degraded to non-steroidal products [18, 19] by *Arthrobacter simplex*. Chenodeoxycholic acid was degraded by *Pseudomonas* spp. N.C.I.B. 10590 to non-steroidal metabolites [16] which were probably of a phenolic nature. The non-steroidal metabolites produced from hyodeoxycholic acid were similar to those isolated from chenodeoxycholic acid degradation. The mechanism of formation of these metabolites is probably similar to the pathway shown by the microbial degradation of androsta-1,4-dien-3,17-dione [20]. In this case 9α-hydroxylation precedes aromatization of the steroid A ring and fission of the C_9-C_{10} bond to give a 9,10-secosteroid. The steroid is then degraded to non-steroidal products.

Table 1. Yield of metabolites with respect to starting material after 18 h incubation

Metabolite	Yield (%)
6α-Hydroxyandrosta-1,4-dien-3,17-dione (**2**)	14.0
Androsta-1,4-dien-3,6,17-trione (**3**)	1.5
6α-Hydroxyandrost-4-en-3,17-dione (**4**)	0.5
6α-Hydroxy-3-oxopregna-1,4-dien-20-carboxylic acid (**5**)	5.0
6α-Hydroxy-3-oxopregn-4-en-20-carboxylic acid (**6**)	4.0



Scheme 1. Proposed microbial degradative pathway of hyodeoxycholic acid. Previously reported metabolites are shown completely enclosed and suggested metabolites are shown in square brackets.

With the above facts taken into consideration a degradative pathway for hyodeoxycholic acid is proposed (Scheme 1).

Compounds **2** and **5** have been produced under anaerobic conditions by an *E. coli* strain isolated from a faecal sample of a colon cancer patient [4]. If hyodeoxycholic acid is to be used for the treatment of gallstones, a study on the carcinogenicity of compounds **2** and **5** would be of value.

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