THE DEGRADATION OF LITHOCHOLIC ACID BY *PSEUDOMONAS* Spp NCIB 10590

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1. Introduction

Reports on the microbial degradation of lithocholic acid show a wide variety of transformations. A series of investigations with a selection of bacteria isolated from the rat intestine [1,2] has shown that many such bacteria have the ability to oxidise and then reduce the 3α-hydroxyl group. Characterised products include 3-oxo-5β-cholan-24-oic acid and 3β-hydroxy-5β-cholan-24-oic acid. Nuclear dehydrogenation of lithocholic acid has also been noted with *Corynebacterium simplex* [3], where 3-oxochola-1,4-dien-24-oic acid was isolated.

The microbial side-chain cleavage of lithocholic acid has been shown with *Corynebacterium simplex* [4] and *Pseudomonas* NCIB 10590 [5] yielding androsta-1,4-dien-3,17-dione in each case. A strain of *Escherichia coli* isolated from a faecal sample of a colon cancer patient has the ability to degrade the side-chain of lithocholic acid yielding both C_{22} and C_{19} products [6].

Microbial degradation of the steroid nucleus has been observed when lithocholic acid was incubated with *Corynebacterium simplex* [3]. In this case a non-steroidal product, namely (4R)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7-aβ-methyl-5-oxindan-1β-yl]valeric acid, was isolated.

We outlined [7] a possible scheme for the microbial degradation of lithocholic acid. The isolation, identification and significance of the products of degradation of lithocholic acid by *Pseudomonas* spp. NCIB 10590 are described in [7].

Abbreviations: GLC, gas–liquid chromatography, TLC, thin-layer chromatography

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2. Materials and methods

2.1. *Reagents*

Lithocholic acid was obtained from Roussel (Paris) while 5α-cholestane, androsta-1,4-dien-3,17-dione, 17β-hydroxyandrosta-1,4-dien-3-one and 17β-hydroxyandrost-4-en-3-one were obtained from Koch Light. General reagents were of Analar grade and obtained from BDH and all solvents were redistilled before use.

2.2. *Chemical analysis*

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. Mass spectra were recorded on an AEI MS 12 spectrometer.

Analysis by gas chromatography was performed at 260°C 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 HP5470 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; by vol.) and the mobilities were measured relative to androsta-1,4-dien-3,17-dione. Products con-

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taining 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]. Acidic products were methylated with diazomethane.

2.3. *Culture conditions, extraction and purification*

The aerobic metabolism of lithocholic acid (1) by *Pseudomonas* NCIB 10590 was carried out in a buffered mineral salts medium comprising: sodium lithocholate (1.0 g); K_2HPO_4 (1.6 g); KH_2PO_4 (0.4 g); KNO_3 (1.0 g); $FeSO_4 \cdot 7\,H_2O$ (2.5 mg); $ZnSO_4 \cdot 7\,H_2O$ (2.5 mg); $MnSO_4 \cdot 4\,H_2O$ (2.5 mg); $MgSO_4 \cdot 7\,H_2O$ (0.1 g); and distilled water to 1 litre (pH 7.2). A 1 litre shake flask culture was incubated at 28°C, the course of the transformation being followed by the direct determination of 1,4-dien-3-one steroids present in the culture. The filtration of samples through a 0.45 μm millipore filter enabled $A_{252\ nm}$ determination of the steroidal mixture. When the $A_{252\ nm}$ value reached a maximum, after 20 h, the culture was terminated by direct extraction of the steroid metabolites into redistilled ethyl acetate (3 × 300 ml). After drying over $MgSO_4$ the solvent was removed under reduced pressure at 50°C to yield 248 mg of a tarry residue. The residue was then taken up in warm dichloromethane (5 ml) and separated by preparative thin-layer chromatography into a series of fractions from which steroids 2, 3, 4 and 5 were crystallised.

2.4. *Metabolites isolated*

2.4.1. Androsta-1,4-dien-3,17-dione (2)

Recrystallisation of (2) from methanol/dichloromethane yielded white prisms (25 mg) m.p. 136–137°C. λ_{max} 244 nm (ϵ 15 000); M^+ 284 ($C_{19}H_{24}O_2$ requires M^+ 284) and *m/e* 122 (1,4-dien-3-one). GLC, R_F 1.4; TLC, R_F 1.00

2.4.2. 17β-hydroxyandrosta-1,4-dien-3-one (3)

Recrystallisation of (3) from methanol/dichloromethane yielded white prisms (3 mg) m.p. 167–168°C. λ_{max} 244 nm (ϵ 14 920); M^+ 286 ($C_{19}H_{26}O_2$ requires M^+ 286), *m/e* 122 (1,4-dien-3-one) and *m/e* 268 (M^+–H_2O). GLC, R_F 1.5; TLC, R_F 0.80.

2.4.3. 17β-hydroxyandrost-4-en-3-one (4)

Recrystallisation of (4) from ethanol/dichloromethane yielded white needles (1 mg) m.p. 152–153°C. λ_{max} 241 nm (ϵ 16 510); M^+ 288 ($C_{19}H_{28}O_2$ requires M^+ 288), *m/e* 124 (4-en-3-one) and *m/e* 270 (M^+–H_2O). GLC, R_F 1.3; TLC, R_F 0.83.

2.4.4. Methyl 3-oxo-pregna-1,4-dien-20-oate (methyl ester of 5)

Recrystallisation of (5) from ethanol/dichloromethane yielded white prisms (11 mg) m.p. 212–214°C. (Found: C,77.61; H,8.90. $C_{23}H_{32}O_3$ requires: C,77.53; H,8.99%). λ_{max} 244 nm. (ϵ 14 950); M^+ 356 ($C_{23}H_{32}O_3$) requires M^+ 356), *m/e* 122 (1,4-dien-3-one) and *m/e* 269 (M^+–side-chain). GLC, R_F 4.1; TLC, R_F, 1.04.

3. Results

The metabolites isolated after 20 h transformation of lithocholic acid [1] are shown in fig.1. The major

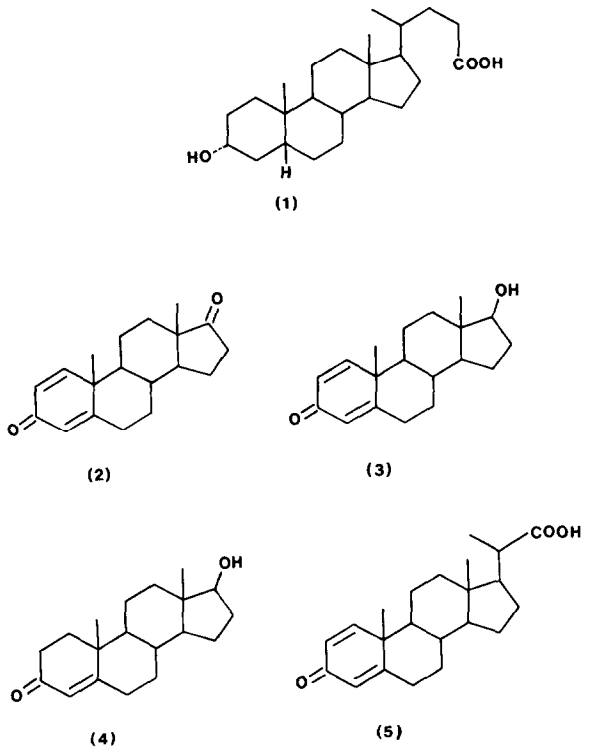

Fig.1. Metabolites isolated from the degradation of lithocholic acid by *Pseudomonas* spp NCIB 10590.

neutral metabolite (2) is identical with androsta-1,4-dien-3,17-dione on thin-layer chromatography, gas chromatography and mass spectrometry and also shows the same melting point. The other two minor neutral metabolites (3,4) are identical with 17β-hydroxyandrosta-1,4-dien-3-one and 17β-hydroxyandrost-4-en-3-one on thin-layer chromatography, gas chromatography and mass spectrometry and also show the same melting points.

The only acidic metabolite (5) was isolated as a crystalline solid. The methyl ester of 5 shows a molecular ion at *m/e* 356 and intense ions at *m/e* 121 and 122 in the mass spectrum, suggesting a steroidal 1,4-dien-3-one A ring structure [9]. Confirmation of this structure is provided by the infrared spectrum (1660 cm^{-1}, 1618 cm^{-1} and 1600 cm^{-1}, αβ-unsaturated ketone) and the ultraviolet spectrum (λ_{max} 244 nm, di-β-substituted αβ-unsaturated ketone, double-bond exocyclic) [10]. Compound (5) resists oxidation, acetylation and reduction suggesting the absence of any hydroxyl or unconjugated ketone groups. The methyl ester of (5) gave rise to an intense ion at *m/e* 269 in the mass spectrum corresponding to the loss of a side-chain from C_{17}. Compound (5) is, therefore, assigned the structure 3-oxo-pregna-1,4-dien-20-carboxylic acid.

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

4. Discussion

The production of testosterone (4) from lithocholic acid marks the first observation of the production of a human hormone by the microbial degradation of bile acids. The microbial degradation of bile acids is potentially of importance in the commercial production of physiologically-active steroids [11].

Table 1
Yield of metabolites with respect to starting material after 20 h incubation

Metabolite	Yield (%)
Androsta-1,4-dien-3,17-dione (2)	9.4
17β-Hydroxyandrosta-1,4-dien-3-one (3)	1.3
17β-Hydroxyandrost-4-en-3-one (4)	0.3
3-Oxo-pregna-1,4-dien-20-carboxylic acid (5)	7.0

Pseudomonas spp. NCIB 10590 has been shown to contain the enzymes necessary for the novel production of physiologically active steroids (3,4). Although lithocholic acid is expensive to use as a substrate it can be produced from chenodeoxycholic acid by 7α-dehydroxylation [12–15] or possibly from cholesterol [16].

The isolation of a C_{22} metabolite (5), as with the degradation of other bile acids by *Pseudomonas* spp. NCIB 10590 [17,18], emphasises that bile acid side-chain cleavage proceeds probably by β-oxidation from a C_{24} bile acid through a C_{22} metabolite to a C_{19} androstane. Such side-chain cleavage has been noted with lithocholic acid under anaerobic conditions [6] with a strain of *E. coli* isolated from a faecal sample of a colon cancer patient. This observation extends the importance of the work to the area of colon cancer, since the microbial degradation of bile acids is implicated in the aetiology of colon cancer [19]. If bacteria can carry out these reactions in vivo then it is possible that physiologically active steroids (3,4) are produced in the human gut. This could mean that gut bacteria are capable of contributing to the level of circulating steroid hormones in the body.

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