Steroid Catechol Degradation: Disecoandrostane Intermediates Accumulated by *Pseudomonas* Transposon Mutant Strains

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Eleven transposon mutant strains affected in bile acid catabolism were each found to form yellow, muconic-like intermediates from bile acids. To characterize these unstable intermediates, media from the growth of one of these mutants with deoxycholic acid was treated with ammonia, then the crude product was methylated with diazomethane. Four compounds were subsequently isolated; spectral evidence suggested that they were methyl 12α-hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oate, methyl 4-aza-12β-hydroxy-9(10)-secoandrosta-1,3,5-triene-9,17-dione-3-carboxylate, methyl 4-aza-9α,12β-dihydroxy-9(10)-secoandrosta-1,3,5-trien-17-one-3-carboxylate and 4α-[3′-propionic acid]-5-amino-7β-hydroxy-7aβ-methyl-3aα,4,7,7a-tetrahydro-1-indanone-δ-lactam. It is proposed that the mutants are blocked in the utilization of such muconic-like compounds as the 3,12β-dihydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid formed from deoxycholic acid. A further mutant was examined, which converted deoxycholic acid to 12α-hydroxyandrosta-1,4-dien-3,17-dione, but accumulated yellow products from steroids which lacked a 12α-hydroxy function, such as chenodeoxycholic acid. The products from the latter acid were treated as above; spectral evidence suggested that the two compounds isolated were methyl 4-aza-7-hydroxy-9(10)-secoandrosta-1,3,5-triene-9,17-dione-3-carboxylate and 4α-[1′α-hydroxy-3′-propionic acid]-5-amino-7aβ-methyl-3aα,4,7,7a-tetrahydro-1-indanone-δ-lactam.

INTRODUCTION

In the initial stage of catabolism of the bile acids by Gram-negative micro-organisms, reactions at the two ends of the bile acid molecule occur concurrently (Fig. 1). This results in the dehydrogenation of the A ring, and in side-chain removal, to produce the epimeric androstane compounds (2) and (3) (Leppik, 1983, and references therein). The B ring is then opened, to give initially the secophenol (4) (Leppik, 1981; Park, 1984), then the secocatechol (5) (Park *et al.*, 1988). This is followed by removal of the A ring carbons (except C-5) as the next, and most degraded, pathway intermediate to have been reported is the δ-lactone of the indane acid (8) (Leppik, 1981).

Recently, transposon mutagenesis studies have begun (Ide *et al.*, 1986; Leppik & Sinden, 1987), and the results obtained support the postulated pathway of Fig. 1. In this laboratory, almost 200 Tn*5* and Tn*5*-Mob transposon mutants affected in bile acid catabolism have now been isolated, from four phenotypically different *Pseudomonas* spp. The mutants were divided into groups, and strains in two of the groups were shown to accumulate either 12β-hydroxyandrostane compounds (3B–D), or secophenols (4B–D), when grown in the presence of the bile acids (1B–D) (Leppik & Sinden, 1987). Two further groups examined both accumulated 12α-hydroxyandrosta-1,4-diene-3,17-dione (2B) from deoxycholic acid (1B), but one group was able to grow on steroid substrates which lacked a 12α-hydroxy group, whereas the single member

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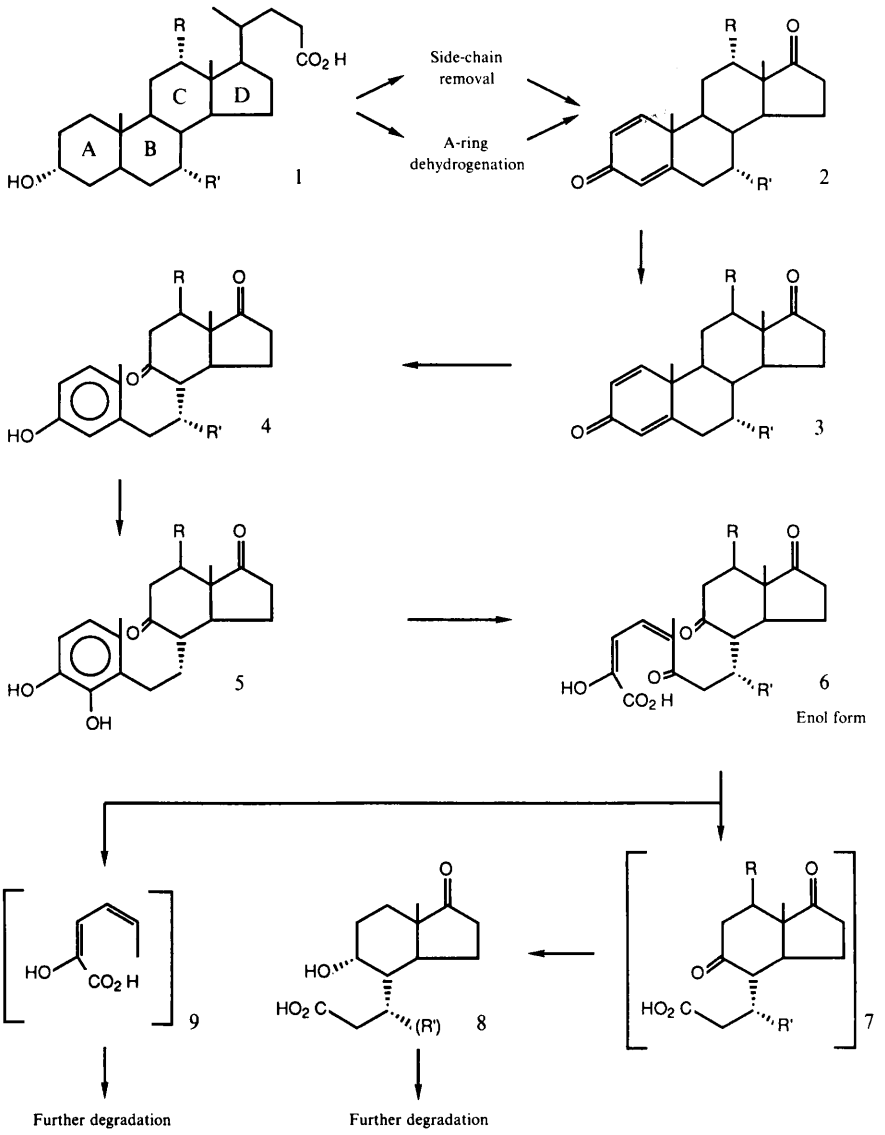

Fig. 1. Abbreviated pathway for the degradation of bile acids by Gram-negative micro-organisms.

A series, R = R′ = H.	1A, lithocholic acid.
B series, R = OH, R′ = H.	1B, deoxycholic acid.
C series, R = H, R′ = OH.	1C, chenodeoxycholic acid.
D series, R = R′ = OH.	1D, cholic acid.

of the second group, RAL8.23, accumulated yellow products from such steroids as chenodeoxycholic acid (1C) (Leppik & Sinden, 1987).

In this paper, a further group of Tn*5* transposon mutants is described, and the yellow disecoandrostane compounds accumulated by one of these mutants, RAL8.17, and also that accumulated by the above mutant, RAL8.23, are characterized. In the accompanying paper, the cellular location of the bile acid catabolic genes is examined, and the cloning of a chromosomal DNA segment coding for at least two of these genes, including the gene mutated in RAL8.17, is described (Leppik, 1989).

METHODS

General. The mutant strains RAL8.17 (Km^r) and RAL8.23 (Km^r) were derived by Tn*5* transposon mutagenesis of the prototrophic *Pseudomonas* sp. RAL8, as previously described (Leppik & Sinden, 1987). The media used, the methods for obtaining spectra and m.p. (melting point) values, and the methods for TLC and column chromatography, were also as previously described (Leppik & Sinden, 1987). Unless otherwise stated, UV spectra were recorded in ethanol, IR spectra in chloroform, and NMR spectra in [^{2}H]chloroform at 100 MHz.

Isolation of the deoxycholic acid products from RAL8.17. RAL8.17 was grown overnight at 25 °C with aeration in LB medium (200 ml) containing kanamycin (50 μg ml^{-1}); this culture was used to inoculate 2 litres of minimal medium containing deoxycholic acid (1B) (10 mM), glucose (5 mM) and yeast extract (0·01%). The mixture was dispensed into six ×2 litre conical flasks, and shaken (200 r.p.m.) at 25 °C. Growth was followed both by TLC examination of ethyl acetate extracts of acidified medium samples, and also by purification of a sample (2 ml) of the medium using a Waters Sep-Pak C_{18} cartridge (Leppik *et al.*, 1982). A portion (100 μl) of the yellow methanol eluate (4 ml) obtained from the Sep-Pak cartridge was diluted into 0·01 M-sodium hydroxide solution (2 ml), then examined spectroscopically at 394 nm. The absorbance reached a maximum after 22·5 h, by which time there was only a low level of precursors remaining, as judged by TLC. The culture was centrifuged to remove the cells, concentrated ammonia solution (220 ml) added to the supernatant, and the mixture left overnight at room temperature. After evaporation to approximately half-volume to remove the ammonia, the mixture was loaded onto an Amberlite XAD-2 column (250 g). The latter was washed with water (1 litre), then with a gradient of 5–12% (v/v) ethanol in water. Those fractions that contained a peak at 270 nm were combined and evaporated. The dried extract (2·62 g) was mixed with ethanol, methylated with diazomethane, then evaporated. The methylated product was purified by passage through a Merck Kieselgel 60 column (85 g), with a gradient of 1–6% (v/v) ethanol in chloroform. Three main fractions were obtained, the second and third of which each appeared to contain essentially one compound. The first fraction was found by TLC to be a mixture of two compounds; these were separated by passage through a second Kieselgel 60 column (50 g), which was eluted with a gradient of 15–100% (v/v) ethyl acetate in hexane. The four compounds obtained are listed below in order of elution, the two compounds from the second column, then the two from the second and third fractions of the first column.

Methyl 12α-hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oate. This compound (90 mg) was recrystallized from an ethyl acetate/hexane mixture to give 17 mg of the title compound, m.p. 243 °C [Literature m.p. 245–7 °C (Leppik, 1983)], with the following properties: $\lambda_{max.}$ 242 nm (ε 19900 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) (KBr), 3520 (OH), 1728 (C=O, ester), 1664 (C=O, 1,4-dien-3-one), 1622, 1604 (C=C, 1,4-dien-3-one); NMR, δ (p.p.m.) 7·00 [1H, d, *J* 11 Hz, C(1)H], 6·23 [1H, d of d, *J*2, 11 Hz, C(2)H], 6·07 [1H, broad s, C(4)H], 4·00 [1H, m, C(12β)H], 3·65 [3H, s, CO_2CH_3], 1·70 [s, C(12α)OH], 1·24 [d, C(21)H_3], 1·22 [s, C(19)H_3], 0·78 [s, C(18)H_3]; mass spectrum, *m/z* 372 (M^+), 354 ($M^+ - H_2O$), 267 ($M^+ - H_2O$ − side chain), 122, 121 (1,4-dien-3-one).

Methyl 4-aza-12β-hydroxy-9(10)-secoandrosta-1,3,5-triene-9,17-dione-3-carboxylate (compound 12B). This compound (0·35 g) was recrystallized from an ethyl acetate/hexane mixture (yield 0·13 g, m.p. 122 °C). It had the following properties: $\lambda_{max.}$ 230 nm (ε 8600 l mol^{-1} cm^{-1}), 272 nm (ε 5700 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) (KBr), 3510 (OH), 1735 [shoulder, C=O, C(17) ketone], 1725 (C=O, ester, 1705 [C=O, C(9) ketone]; NMR, δ (p.p.m.) 7·88 [1H, d, *J* 9 Hz, C(1)H or C(2)H], 7·55 [1H, d, *J* 8 Hz, C(1)H or C(2)H], 4·13 [1H, d of d, *J* 6, 12 Hz, C(12α)H], 3·97 [3H, s, CO_2CH_3], 3·05 [broad s, C(12β) OH], 2·46 [3H, s, C(19)H_3], 1·20 [3H, s, C(18)H_3]; mass spectrum (Fig. 3), parent ion at *m/z* 359·1730 (calculation for $C_{20}H_{25}O_5N$ 359·1733).

Methyl 4-aza-9α,12β-dihydroxy-9(10)-secoandrosta-1,3,5-trien-17-one-3-carboxylate (compound 15, Fig. 4). This compound (70 mg) was recrystallized from an ethyl acetate/hexane mixture (yield 15 mg, m.p. 184–6 °C). It had the following properties: $\lambda_{max.}$ 229 nm (ε 7900 l mol^{-1} cm^{-1}), 273 nm (ε 5400 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) (KBr) 3400 (OH), 1728 [C=O, C(17) ketone], 1718 (C=O, ester); NMR, δ (p.p.m.) 7·90 [1H, d, *J* 9 Hz, C(1)H or C(2)H], 7·58 [1H, d, *J* 9 Hz, C(1)H or C(2)H], 4·23 [1H, d of d after D_2O exchange, *J*5, 14 Hz, C(12α)H], 3·98 [3H, s, CO_2CH_3], 3·82 [1H, m, C(9β)H], 2·38 [3H, s, C(19)H_3], 0·95 [3H, s, C(18)H_3]; mass spectrum (Fig. 4), parent ion at *m/z* 361·1893 (calculation for $C_{20}H_{27}O_5N$ 361·1889).

4α-[3'-Propionicacid]-5-amino-7β-hydroxy-7aβ-methyl-3aα,4,7,7a-tetrahydro-1-indanone-δ-lactam(compound 14B). This compound (0·22 g) was recrystallized from ethyl acetate [yield 80 mg, m.p. 241·5 °C (decomposition)]. It had the following properties: $\lambda_{max.}$ 230 nm (ε 16100 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) (KBr) 3550 (OH or NH), 1726 [C=O, C(1) ketone], 1654 (C=O), lactam); NMR, δ (p.p.m.) 7·47 [1H, broad s, NH], 4·77 [1H, d of d, *J*2, 2 Hz, C(6)H], 4·49 [1H, d of d, *J*2, 2 Hz, C(7α)H], 1·02 [3H, s, C(7aβ)CH_3]; mass spectrum (Fig. 5), parent ion at *m/z* 235·1202 (calculation for $C_{13}H_{17}O_3N$ 235·1208).

Isolation of the chenodeoxycholic acid products from RAL8.23. Growth conditions were as for RAL8.17, except that chenodeoxycholic acid (1C) (5 mM) replaced deoxycholic acid, the pH of the minimal medium was raised from 7·4 to 8·2, and the ammonium chloride concentration in the minimal medium was increased from 19 mM to 60 mM. After 20 h, the absorption at 390 nm was dropping, and there was only a low level of precursors remaining, as judged by TLC, so the culture was centrifuged and concentrated ammonia solution (220 ml) added to the supernatant; the mixture was then left at room temperature overnight. Excessive frothing prevented evaporation

of the mixture to half-volume, so the mixture was adjusted to pH 7·0 with glacial acetic acid, then purified using Amberlite XAD-2 column chromatography, as described above, except that a gradient of 2–100% (v/v) methanol in water was used. The fractions with an absorbance maximum at 270 nm were combined, dried, dispersed in aqueous methanol, then methylated with diazomethane. The methylated product (2·91 g) was purified by passage through a Merck Kieselgel 60 column (85 g), with a gradient of 1–6% (v/v) ethanol in chloroform. Two main compounds were obtained, which are described below in order of elution.

Methyl 4-aza-7-hydroxy-9(10)-secoandrosta-1,3,5-trien-9,17-dione-3-carboxylate (compound 12C). This compound (0·59 g) was recrystallized from an ethyl acetate/hexane mixture [yield 0·45 g, m.p. 150–150·5 °C (decomposition)]. It had the following properties: $\lambda_{max.}$ 230 nm (ε 8900 l mol^{-1} cm^{-1}), 272 nm (ε 5700 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) 3740 (OH), 1736 [C=O, C(17) ketone], 1720 (C=O, ester), 1704 [C=O, C(9) ketone]; NMR, δ (p.p.m.) 7·91 [1H, d, *J* 8 Hz, C(1)H or C(2)H], 7·59 [1H, d, *J* 9 Hz, C(1)H or C(2)H], 5·39 [1H, d, *J* 4 Hz, C(7)OH], 4·56 [1H, m, C(7)H], 3·95 [3H, s, CO_2CH_3], 2·38 [3H, s, C(19)H_3], 1·14 [3H, s, C(18)H_3]; mass spectrum, parent ion at *m/z* 359·1689 (calculation for $C_{20}H_{25}O_5N$ 359·1733).

4α-[1′-Hydroxy-3′-propionic acid]-5-amino-7aβ-methyl-3aα,4,7,7a-tetrahydro-1-indanone-δ-lactam (compound 14C). This compound (0·19 g) was recrystallized from ethanol [yield 60 mg, m.p. 285 °C (decomposition)]. It had the following properties: $\lambda_{max.}$ 228 nm (ε 14900 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) 3610 (OH or NH), 1730 [C=O, C(1) ketone], 1672 (C=O, lactam); NMR, δ (p.p.m.) 7·43 (1H, broad s, NH), 5·05 [1H, m, C(6)H], 4·29 [1H, m, C(1′β)H], 2·74, 2·71 [2H, 2s, C(2′)H], 0·97 [s, 7aβCH_3]; mass spectrum, parent ion at *m/z* 235·1207 (calculation for $C_{13}H_{17}O_3N$ 235·1208).

RESULTS

Mutant strains that form a disecoandrostane compound (6B) from deoxycholic acid (1B)

Eleven transposon mutant strains were isolated which turned the medium yellow when grown in the presence of a bile acid. All produced slight growth when plated onto minimal medium containing either deoxycholic acid (1B) or 12α-hydroxy-3-oxochola-1,4-dien-24-oic acid, and negligible growth on plates containing either 3α-hydroxy-5β-androstan-17-one or androsta-1,4-diene-3,17-dione (3A). This suggested that the mutants were blocked in ring degradation (Leppik & Sinden, 1987).

The eleven strains were each grown in minimal medium containing glucose (5 mM) and deoxycholic acid (2·5 mM), and samples taken at 24 h. The samples were acidified, extracted with ethyl acetate, and the extracts examined by TLC. No spots could be seen for any strain, except for a dark spot at the origin. Samples were also purified via a Waters Sep-Pak C_{18} cartridge (Leppik *et al.*, 1982). The yellow methanol eluates, after dilution into 0·01 M-sodium hydroxide solution, showed $\lambda_{max.}$ values of 393–396 nm. These maxima are in the same position as that reported for 3-hydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid (6A) (Gibson *et al.*, 1966), suggesting that the eleven mutant strains accumulated the analogous compound (6B) from deoxycholic acid (1B).

Stability of the yellow product formed from deoxycholic acid

The disecoandrostane compound studied by Gibson *et al.* (1966) was reported to be unstable, so the stability of the product from deoxycholic acid was examined, as a prelude to its characterization. One of the mutant strains, RAL8.17, was grown for 24 h in minimal medium containing glucose (5 mM) and deoxycholic acid (10 mM), by which time the compounds absorbing at 394 nm were at about their maximum concentration. The yellow medium was centrifuged, the supernatant filter-sterilized, and then a few drops of chloroform were added to ensure sterility. Samples were taken at various times, diluted into 0·01 M-sodium hydroxide solution, then read at 394 nm. It was found that the absorbance at 394 nm dropped at the rate of approximately 13% per hour, showing that there would be an approximately 50% loss in the yellow products in 5 h.

Isolation of the intermediates formed by RAL8.17 from deoxycholic acid

Because of the instability of the products, it was necessary to convert them into more stable compounds. One method of achieving this could be by reaction with ammonia, as Gibson *et al.* (1966) had shown that the disecoandrostane compound (6A) reacts with ammonia to form a stable pyridine acid (11A) (Fig. 2), the latter having a $\lambda_{max.}$ of 273 nm.

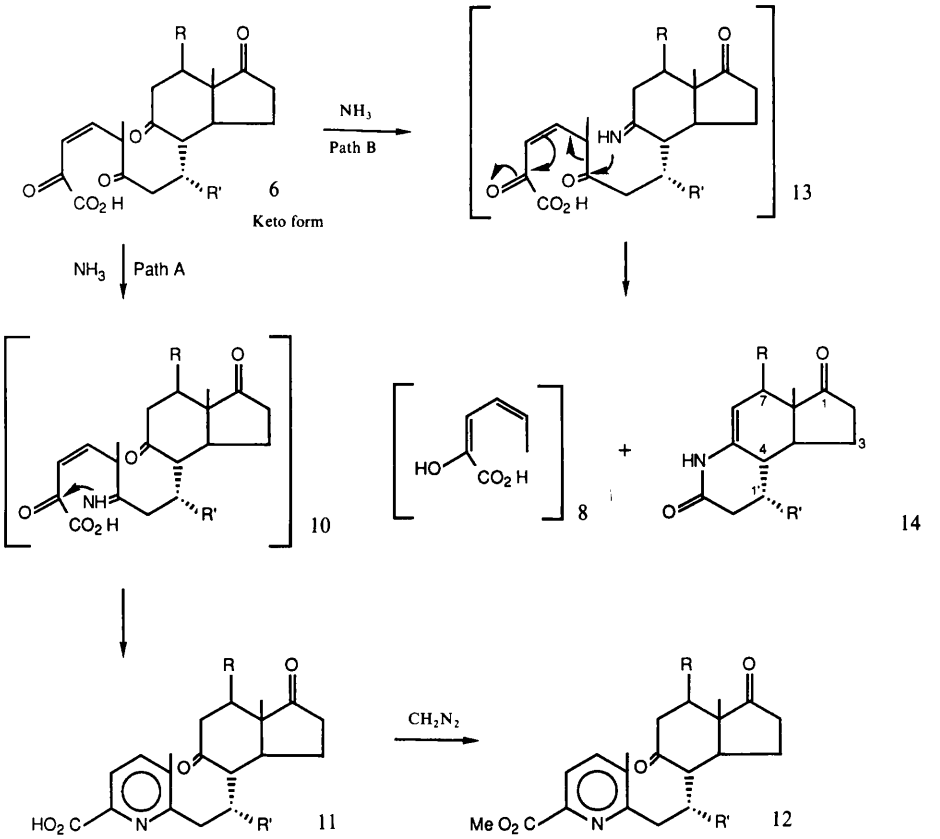

Fig. 2. Proposed scheme for the reactions between the disecoandrostane (compound 6) and ammonia. A–D series, as for Fig. 1.

Strain RAL8.17 was grown in minimal medium containing glucose (5 mM) and deoxycholic acid (10 mM), and growth was followed as described above. The absorbance at 394 nm reached a maximum after 22·5 h, when the culture was centrifuged, concentrated ammonia solution added to the supernatant, and the mixture left at room temperature. After 20 h, the 394 nm peak had disappeared, and a shoulder had appeared at 270 nm. The mixture was evaporated to approximately half-volume to remove excess ammonia, then purified by XAD-2 column chromatography. Those fractions with an absorbance maximum at 272 nm were combined, dried, mixed with ethanol, and treated with diazomethane. The methylated products were then purified by silica gel column chromatography, to yield four main products.

Spectral examination of the first compound to be eluted from the silica gel column indicated that it contained a 1,4-dien-3-one function [$\lambda_{max.}$ 242 nm (ε 19900 l mol^{-1} cm^{-1}); NMR, δ (p.p.m.) 7·00 [1H, d, J 11 Hz, C(1)H], 6·23 [1H, d of d, J2, 11 Hz, C(2)H], 6·07 [1H, broad s, C(4)H)], a 12α-hydroxy function [NMR, δ 4·00 (1H, m, band shape consistent with that for a βH associated with a 12α-hydroxy function), 1·70 (s, disappears after D_2O exchange)], and a three-carbon side chain [mass spectrum, m/z 372 (M^+), 267 ($M^+ - H_2O$ – side chain (87)]. This suggested that the compound was methyl 12α-hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oate, and this was confirmed by comparison with the compound previously isolated from RAL8 (Leppik, 1983).

The spectral data of the second compound to be eluted indicated that it was the pyridine methyl ester compound (12B). Thus, it had the following properties: $\lambda_{max.}$ 230 nm (ε 8600 l mol^{-1} cm^{-1}), 272 nm (ε 5700 l mol^{-1} cm^{-1}); $IR_{max.}$ (cm^{-1}) 1725 (ester); NMR, δ (p.p.m.) 7·88 [1H, d, J 9 Hz, C(1)H or C(2)H], 7·55 [1H, d, J 8 Hz, C(1)H or C(2)H], 3·97 (3H, s, CO_2CH_3), 2·46 [3H, s,

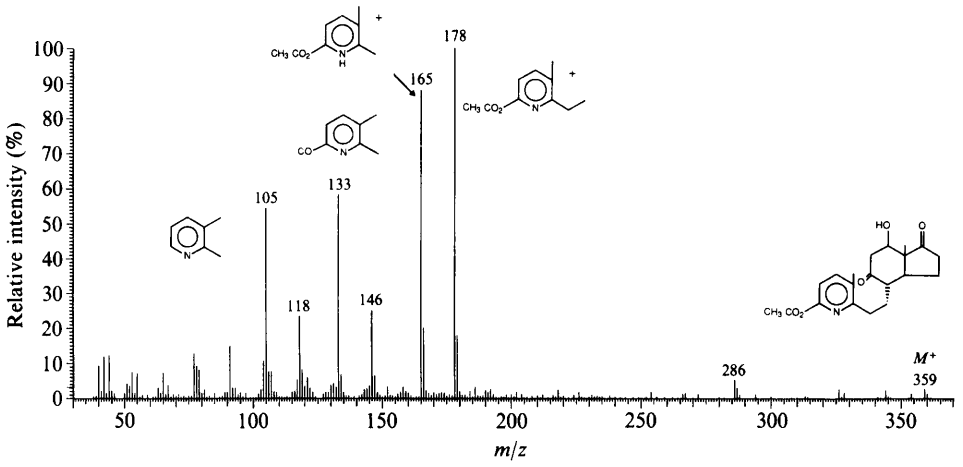

Fig. 3. Mass spectrum of the pyridine ester (12B).

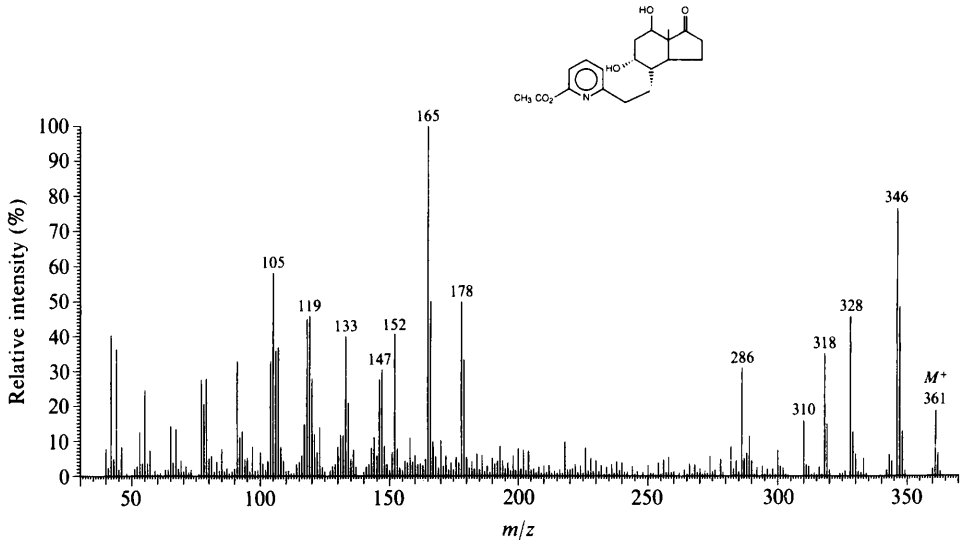

Fig. 4. Mass spectrum of the pyridine ester (15).

C(19)H_3)]. Additionally it contained two carbonyl functions [$IR_{max.}$ 1735 cm^{-1} (shoulder, C(17) ketone), 1705 cm^{-1} (C(9) ketone)], and a 12β-hydroxy function [NMR, δ 4·13 (1H, d of d, J6, 12 Hz, C(12α)H), 3·05 (broad s, disappears after D_2O exchange)]. The structural assignment was supported by the mass spectral data (Fig. 3), which showed a parent ion at m/z 359·1730, which corresponds to $C_{20}H_{25}O_5N$, and a base peak at m/z 178·0872, which corresponds to $C_{10}H_{12}O_2N$.

The third compound to be eluted also appeared to be a pyridine methyl ester (relevant spectral data similar to that of compound 12B) but appeared to contain only one carbonyl function [$IR_{max.}$ 1728 cm^{-1} (C(17) ketone)], but two hydroxy functions [NMR, δ (p.p.m.) 4·23 (1H, d of d after D_2O exchange, J5, 14 Hz, C(12α)H), 3·82 (1H, broad s, C(9β)H, same band shape as the C(9β)H of the δ lactone of compound 8A)]. This is compatible with the compound being methyl 4-aza-9α,12β-dihydroxy-9(10)-secoandrosta-1,3,5-trien-17-one-3-carboxylate (compound 15, Fig. 4). This assignment is supported by the mass spectrum (Fig. 4), which showed a parent ion at m/z 361·1893, which corresponds to $C_{20}H_{27}O_5N$. The difference in the mass spectral patterns

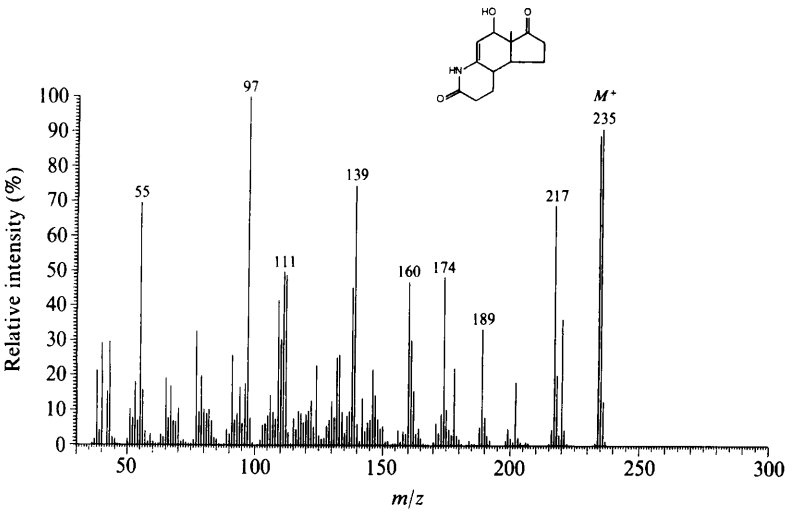

Fig. 5. Mass spectrum of the δ-lactam (14B).

between secosteroids which contain a 9-keto function, such as compound (12B) (Fig. 3), and the secophenol compound (4B) (Leppik, 1981), and those that contain a 9-hydroxy function, such as compound (15) (Fig. 4), is striking, and is indicative of the strong influence that the 9-keto function has on the fragmentation pattern.

High-resolution mass spectral examination of the final compound to be eluted showed a parent ion at m/z 235·1202, which corresponds to $C_{13}H_{17}O_3N$. This, and the NMR data in particular, indicated that the compound could be the δ-lactam compound (14B) [NMR, δ 7·47 (1H, broad s, disappears after D_2O exchange, NH), 4·77 (1H, d of d, J2, 2 Hz, C(6)H), 4·49 (1H d of d, J2, 2 Hz, C(7α)H), 1·02 (s, 7aβCH_3). This structural derivation is supported by comparison with the spectral data published for the δ-lactam (14A) by Hayakawa *et al.* (1976). In particular, compound (14A) is reported to have a $\lambda_{max.}$ at 230·5 nm (ε 13800 l mol^{-1} cm^{-1}), and the compound isolated here (14B) has a $\lambda_{max.}$ at 230 nm (ε 16100 l mol^{-1} cm^{-1}).

Attempt to increase the product yield

After growth of RAL8.17 as described above, the yields of the main products obtained by silica gel chromatography were quite low – 5·1% for the pyridine derivative (12B), and 4·8% for the lactam (14B). Whilst this is largely a reflection of the instability of the disecoandrostane (6B), it was considered that the yield of products might be improved by increasing the concentration of free ammonia in the medium. This could be achieved both by raising the pH, and by raising the concentration of ammonium chloride. Tests showed that the parent strain RAL8 could grow satisfactorally at pH values up to 8·2, and at ammonium chloride concentrations up to 60 mM, as against the normal M9 medium of pH 7·4 and 19 mM-ammonium chloride. This would result in an increase in the free ammonia concentration from approximately 0·2 mM to approximately 6 mM. Preliminary experiments with RAL8.17 indicated that at pH8·2 and 60 mM-ammonium chloride, there was an approximate two fold increase in the product yield of the pyridine derivative (12B), so these conditions were used in the growth of RAL8.23 described below.

Isolation of the intermediates formed by RAL8.23 from chenodeoxycholic acid

Strain RAL8.23 has been previously reported to convert deoxycholic acid (1B) into 12α-hydroxyandrosta-1,4-diene-3,17-dione (2B), but to form a yellow product when grown with chenodeoxycholic acid (1C) (Leppik & Sinden, 1987). To identify the yellow product, RAL8.23 was grown in minimal medium, pH 8·2, containing glucose (5 mM), chenodeoxycholic acid (5 mM) and ammonium chloride (60 mM). Growth was monitored, and the products isolated, as described for RAL8.17. Two main compounds were isolated.

The first compound to be eluted from the silica gel column was found to be a pyridine methyl ester, and to contain two carbonyl functions (relevant spectral data similar to that of compound 12B). The compound also contained an hydroxy function ($IR_{max.}$ 3740 cm^{-1}; NMR, δ (p.p.m.) 5·39 (1H, d, J 4 Hz, disappears after D_2O exchange, C(7)OH), 4·56 (1H, m, C(7)H)]. These results are compatible with the compound being the pyridine ester (12C). This is supported by the mass spectrum, which showed a small parent ion at m/z 359·1689, which corresponds to $C_{20}H_{25}O_5N$. The mass spectrum contained four major peaks, three of which, at m/z 105, 133 and 165, were at the same positions as that obtained for compound (12B) (Fig. 3). The fourth major peak, at m/z 194·0819, corresponds to $C_{10}H_{12}O_3N$, and could be equivalent to the m/z 178 peak of compound (12B), but containing the extra oxygen atom originally present at C(7) in chenodeoxycholic acid (1C).

The second compound to be eluted had a parent ion in the mass spectrum at m/z 235·1207, which corresponds to $C_{13}H_{17}O_3N$. Thus the compound could be the δ-lactam (14C); this is supported by comparison of the spectral data with that of the δ-lactam (14B), described above. Thus, it has a $\lambda_{max.}$ at 228 nm (ε 14900 l mol^{-1} cm^{-1}), and NMR peaks at δ 7·43 (1H, broad s, disappears after D_2O exchange, NH), 5·05 [1H, m, C(6)H], 4·29 [1H, m, C(1′β)H, same band shape as that found in 7α,12α-dihydroxyandrosta-1,4-diene-3,17-dione (2D)], 2·74, 2·71 [2H, 2s, C(2′)H], 0·97 [s, C(7aβ)CH_3].

The yield of the products obtained in this experiment was found to be higher than that obtained after growth of RAL8.17, and may reflect the increased concentration of free ammonia in the medium. Thus, the yields, after isolation, of the pyridine derivative (12C) and the δ-lactam (14C) were 17% and 8·4%, respectively compared with 5·1% for the pyridine derivative (12B) and 4·8% for the δ-lactam (14B), as reported above.

DISCUSSION

The microbial degradation of aromatic compounds frequently occurs via their conversion into phenolic, then catecholic, intermediates, followed by the cleavage of the aromatic ring (Bayly & Barbour, 1984). This mode of degradation is also found as part of the steroid catabolic pathway, where the rupture of the B ring (Fig. 1) initially produces phenolic secosteroids such as compound (4). The first report of such secosteroids was by Dodson & Muir (1958), who isolated the secophenol 4A after fermentation of androst-4-ene-3,17-dione by a *Pseudomonas* sp. Similar secophenols have been isolated from other androstane substrates (Schubert *et al.*, 1960; Wang & Sih, 1963), from progesterone (Schubert *et al.*, 1961), and from bile acids (Leppik, 1981; Park, 1984).

Evidence that the phenolic secosteroids were then hydroxylated to catecholic secosteroids was obtained by Sih *et al.* (1966), who synthesized the secocatechol (5A), and demonstrated its conversion into the indane acid (7A) by cell extracts of *Nocardia restrictus*. Direct evidence for the intermediacy of secocatechols, however, has only recently been obtained, when it was found that certain *P. putida* mutant strains would accumulate the secocatechol (5D) when grown in the presence of cholic acid (1D) (Park *et al.*, 1988).

The cleavage of catechol rings can occur either between the two hydroxy groups (*ortho*-cleavage), or to one side of the diol grouping (*meta*-cleavage). Of the two, *meta*-cleavage has been found to be used for the degradation of a wider range of compounds than has *ortho*-cleavage (Bayly & Barbour, 1984). Evidence that steroid secocatechols also undergo *meta*-cleavage was obtained by Gibson *et al.* (1966), who demonstrated that cell extracts of *N. restrictus* could convert the synthetic secocatechol (5A) into the disecoandrostane (6A), the latter being isolated as its pyridine derivative (11A).

The results presented in this paper show that *meta*-cleavage of the aromatic A ring is also an integral part of the bile acid degradative path. Thus, eleven transposon mutant strains, isolated from four phenotypically different *Pseudomonas* spp., were each found to accumulate a yellow product from deoxycholic acid (1B). The UV absorption spectra suggested that this product could be the disecoandrostane (6B). This was confirmed for one of the mutants, RAL8.17, where the major yellow compound present was isolated as the pyridine methyl ester derivative (12B).

A further mutant strain, RAL8.23, was also examined. This strain had been reported to produce 12α-hydroxyandrosta-1,4-diene-3,17-dione (2B) from deoxycholic acid (1B), but to form yellow products from steroids which lack a 12α-hydroxy function (Leppik & Sinden, 1987). The yellow product from chenodeoxycholic acid (1C) has now been shown to be the 7-hydroxy-disecoandrostane (6C), this again being isolated as its pyridine derivative (12C). Strain RAL8.17 also accumulates a yellow product from chenodeoxycholic acid, which has the same ultraviolet spectrum as the compound produced by RAL8.23 (unpublished data). Together with the fact that the two mutants do not cross-feed on minimal medium plates containing chenodeoxycholic acid (unpublished data), this suggests that both mutants accumulate the same yellow product from this bile acid. However, the genetic relationship between these two strains has yet to be clarified.

With secocatechols in general, further degradation following ring rupture can occur via a hydrolytic step, or via a three-step dehydrogenase path (Bayly & Barbour, 1984; Harayama *et al.*, 1987). With the steroidal secocatechols, however, there is no oxidizable aldehyde function in the 9-position, so the only pathway which can be utilized is the one-step hydrolytic path, as shown in Fig. 1, to yield compound (9) as the A ring product. Compound (9) has not been reported as a bile acid catabolite, but has been shown by Coulter & Talalay (1968) to be an intermediate in the degradation of androst-4-ene-3,17-dione by *P. testosteroni.*

The other major products reported here are the lactams (14B) and (14C). These could have been formed either from the disecoandrostane compounds (6B) or (6C), as shown in path B, Fig. 2, or formed from degradation products of (6B) and (6C). A third possibility, that they were formed by the reaction of ammonia with (7B) or (7C), is considered unlikely, as (7B) has been isolated in connection with other work (unpublished data), and no compound with the same R_F on TLC as (7B) has been detected in any deoxycholic acid fermentation by the twelve mutants examined in this paper. The related lactam (14A) has been isolated by Hayakawa *et al.* (1976) from a cholic acid (1D) fermentation by *Streptomyces rubescens.* They postulated that this lactam was formed by reaction of ammonia with the postulated intermediate indane acid (7A). This was subsequently confirmed, when lactam (14A) was isolated from a fermentation of the indane acid (7A) by *S. rubescens* (Hashimoto & Hayakawa, 1977). However, as discussed above, it is not considered to be the route for the formation of the lactams (14B) or (14C) in the current work.

It is of interest that both the 7- and 12-hydroxy functions present in cholic acid (1D) are absent in the lactam (14A) produced by *S. rubescens*, whereas the two hydroxy groups are retained in the lactams (14B) and (14C) produced by the *Pseudomonas* mutants. This is a reflection of the different degradative paths used by Gram-positive and Gram-negative micro-organisms. With Gram-positive micro-organisms, the 7-hydroxy function is lost early in the degradative path, via the sequence: 3α,7α-dihydroxy → 7α-hydroxy-3-one → 7α-hydroxy-4-en-3-one → 4,6-dien-3-one → 4-en-3-one → 1,4-dien-3-one (Hayakawa, 1973). Less is known, though, about the fate of the 12-hydroxy group in the Gram-positive path. It is initially oxidized to a ketone, possibly to avoid steric hinderance of the 9α-hydroxylation reaction. However, the point at which the 12-keto function is then lost is unknown, it being present in the dinorcholanic acid intermediates isolated, but absent from the indane compounds (Hayakawa, 1973). This is in contrast to the Gram-negative *Pseudomonas* spp. studied, where both the 7-hydroxy and 12-hydroxy functions are retained at least as far as the disecoandrostanes (6B) and (6C).

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