# PHENOLIC 9,10-SECOSTEROIDS AS PRODUCTS OF THE CATABOLISM OF BILE ACIDS BY A PSEUDOMONAS SP.

R.J. PARK

CSIRO Division of Food Research, Meat Research Laboratory, P.O. Box 12, Cannon Hill, 4170, Australia

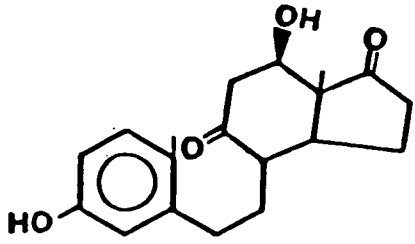
Received 1-10-85.

ABSTRACT

The obligate aerobe, Pseudomonas putida ATCC 31752, efficiently utilises bile acids as a source of carbon and energy for growth and maintenance. When aeration is considerably restricted, a consequence to the catabolism of the bile acids in a fermentor is an accumulation of certain steroidal catabolites. Evidence is presented to show that among these are hydroxy-9,10-seco-1,3,5(10)-androstratriene-9,17-diones and those from four of the common bile acids, cholic, chenodeoxycholic, hyodeoxycholic and deoxycholic acids have been isolated and their structures determined. The product of catabolism of hyodeoxycholic acid appears to exist in a hemi-acetal form which readily forms an acetal during isolation procedures. All but one of these are described for the first time.

## INTRODUCTION

The microbial degradation or catabolism of bile acids to supply carbon and energy for growth of numerous microorganisms, largely of gram positive genera, has been reviewed by Hayakawa (1,2). The catabolism of bile acids by the rapidly growing and obligately aerobic Pseudomonas has been the subject of more recent studies by a group from Liverpool (U.K.)(3-7) and our laboratory (8-12). Leppik (8-10) isolated a large number of catabolites from a fermentor growth of Pseudomonas sp. ATCC 31753 (formerly Pseudomonas MR108) with deoxycholic acid as its sole carbon source. This allowed the construction of a pathway for the catabolism of deoxycholic and other bile acids by this and other Pseudomonas spp. (10). Part of this pathway proposed for the degradation or catabolism of bile acids is shown in Fig. 1. Among these catabolites of deoxycholic acid, but isolated in low yield (8), was 3,12β-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione I.




I

This was the first phenolic 9,10-secosteroid isolated as a catabolite of bile acids, although Tenneson et al (4-6) and Bilton et al (7) found some evidence for the presence of phenols amongst the catabolites of bile acids by Pseudomonas sp. NC1B 10590. In 1961, Dodson and Muir (13) obtained 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione by incubation of 4-androstene-3,17-dione with a Pseudomonas sp. and recently the same secosteroid was isolated in fair yield by the catabolism of cholesterol by a blocked mutant of Mycobacterium fortuitum (14).

When Pseudomonas sp. ATCC 31752, now recognised as a strain of P.putida (15), was used to transform the mixed bile acids of ox bile under conditions of limited aeration, the corresponding hydroxy-1,4-androstadiene-3,17-diones (for convenience these are subsequently referred to as hydroxy androstadienedione compounds) were obtained in good yield (11). These catabolites immediately precede the phenolic secosteroids as stable catabolites in the degradative pathway in Fig. 1. However, no phenolic secosteroids were isolated

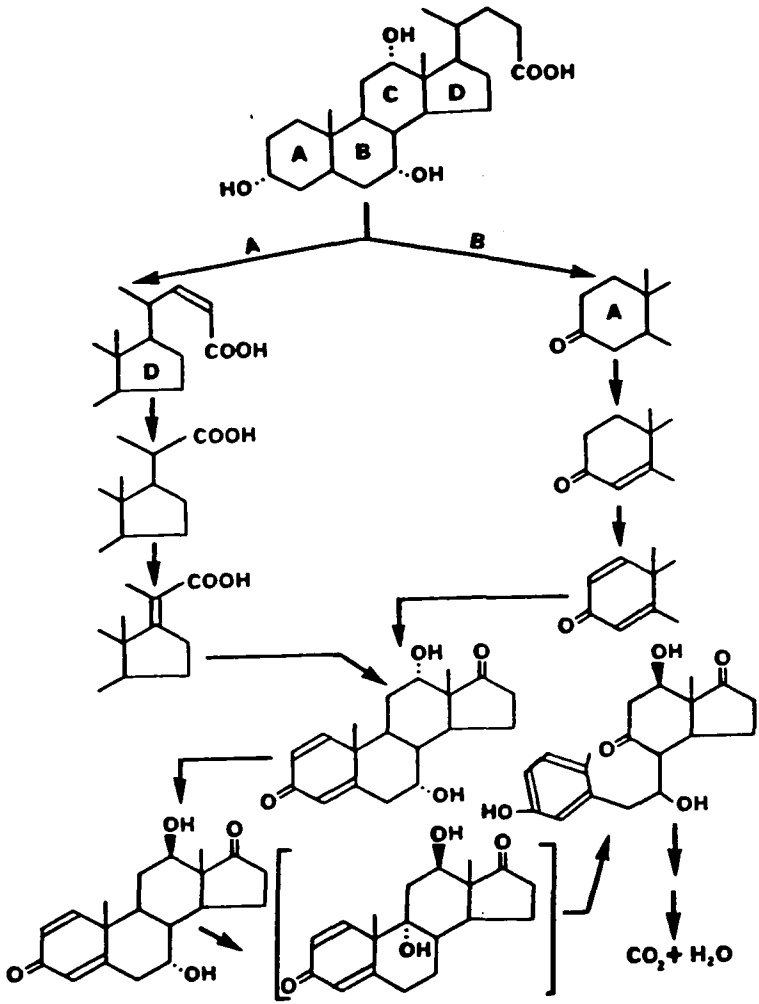
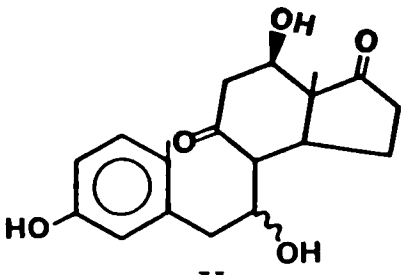



FIGURE 1: Pathway proposed for the catabolism of cholic acid by Pseudomonas spp. Pathways A and B represent the apparently independent routes for sidechain shortening and A-ring functionalization.

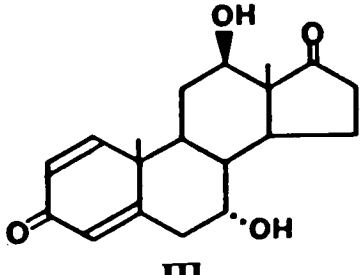
in this study, the biotransformation being terminated as soon as the hydroxy androstadienedione compounds had reached their maximum concentration. The phenolic secosteroids are of some interest to us in our endeavours to discover useful compounds which can be produced from bile acids of slaughtered livestock. In the present study, the bile acids were catabolised by growing or fully grown cells of P.putida ATCC 31752 in a fermentor under limiting aeration, but carried beyond the point where the appropriate hydroxy androstadienedione compound is the sole product and the novel products isolated and identified.

## RESULTS AND DISCUSSION

The fermentor catabolism of cholic acid by Pseudomonas putida ATCC 31752, using extremely limited aeration, was terminated when HPLC and UV analyses indicated the presence of a considerable amount of a component with an absorption maximum near 280nm. At this time, the concentration of 7α,12β-dihydroxyandrostadienedione III was dropping rapidly and cholic acid was absent. Silica gel chromatography of the recovered products gave a pure compound II which showed a high resolution mass spectrum indicating a molecular formula of $C_{19}H_{24}O_5$ and which possessed spectral properties of a methyl alkyl phenol similar to I and IR evidence for the presence of cyclopentanone and cyclohexanone carbonyl groups. The low resolution mass spectrum showed a distinctive pattern of intense peaks at m/z values of 121, 122, 150, 182, and 193, consistent with splitting between C6 and C7 or C7 and C8 (see Fig. 2), analogous to




II




III

the corresponding spectrum of 7α-12β- dihydroxyandrostadienedione III (4). The PMR spectrum of II (Table 1) was similar to that of I, with the exception that the unresolved multiplet centred at δ 4.04 arose from two protons (C7 and C12) instead of one in I (C12). This signal was similar to that in 7α,12β-dihydroxyandrostadienedione arising from the 7β and 12α protons, which however was resolvable into 2 groups of signals unlike that in II (4,11). The ready loss of 2 molecules of water in the mass spectrum confirmed the presence of the two non-aromatic hydroxyl groups in positions favorable for elimination.

TABLE 1

The δ values of some significant protons from the 100 MHz $^1$H NMR of five phenolic 9,10-secosteroids[a] obtained from the degradation of bile acids by Pseudomonas putida ATCC 31752.

| | δ value of proton(s) at carbon | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9,10-secosteroid[a] | 1 | 2 | 4 | 6 | 7 | 12 | 18 | 19 |
| 3,12β-dihydroxy (I) | 6.94 | 6.58 | 6.65 | - | - | 4.03 | 1.20 | 2.23 |
| 3,7,12β-trihydroxy (II) | 6.93 | 6.56 | 6.68 | - | 4.04[b] | 4.04[b] | 1.07 | 2.21 |
| 3,7-dihydroxy (IV) | 6.98 | 6.58 | 6.63 | 2.95 | 3.96 | - | 1.06 | 2.23 |
| 3,6-dihydroxy (VI) | 6.92 | 6.58 | 7.35 | 5.32 | - | - | 0.93 | 2.18 |
| 3-hydroxy-9-methoxy (VII) | 6.97 | 6.62 | 7.03 | 5.35 | - | - | 0.95 | 2.27 |

[a] di- or tri-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione

[b] unresolved

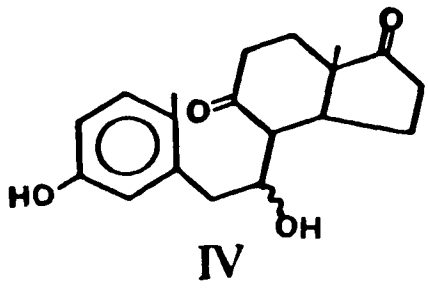
This evidence points to the structure 3,7,12β-trihydroxy-9,10-seco-1,3,5 (10)-androstatriene-9,17-dione II for this compound.

The stereochemistry of II at the C8 position is assumed to be unchanged from that of its immediate precursor, 7α,12β-dihydroxy-1,4-androstadiene-3,17-dione or cholic acid, i.e. 8β-H. Neither the 9α-hydroxylation nor the retro-aldol rearrangement involved in formation of II by the microorganism should require a configuration change at the C-8 position, while the procedures employed in its isolation are probably sufficiently mild to leave this position unaffected, despite the presence of the carbonyl group at C9. Although the opening of the steroidal B ring to form II has removed the need or ability to specify the conformation of the C7 hydroxyl group, C7 remains asymmetric. A change in configuration at C7 is

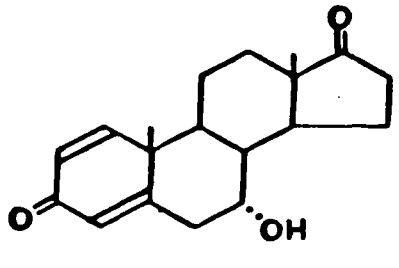
considered unlikely, due to the apparent lack of need for the microorganism to effect such a change, unlike the corresponding C12 hydroxyl (10). However, in the absence of any evidence, no assignment can be made at this position. The full name of 3,7ζ,12β-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione is proposed for II. The yield of II (ca. 11.7% of theoretical) was much greater than that of the correponding phenolic secosteroid from deoxycholic acid (ca. 0.25% theoretical) (8), attributed to the differing fermentor conditions rather than the different Pseudomonas spp. An even higher yield of II would have been expected if the bile acid had been transformed by fully grown cells, as some of the bile acid would have been used to provide carbon for growth of the cells in the fermentor.

A transformation of deoxycholic acid to 3,12β-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione I was carried out using P.putida ATCC 31752 cells which had been grown on glycerol as carbon source. The bile acid was added to the fermentor when the glycerol was fully utilised (indicated by a sharp increase in the dissolved oxygen concentration in the fermentor culture). In this instance compound I was obtained in a yield about 72% of theoretical, which could have been improved to about 90% if the fermentor process had been terminated 1-2h earlier (estimated by HPLC analysis). This procedure provides a fairly efficient method for the biotransformation of deoxycholic acid to this phenolic secosteroid.

In the biotransformation of chenodeoxycholic acid to a phenolic 9,10-secosteroid by P.putida ATCC 31752 cells grown on glycerol, a much less efficient conversion resulted than with deoxycholic acid.




IV




V

HPLC and TLC analyses of samples periodically withdrawn from the fermentor revealed that chenodeoxycholic acid had been completely exhausted 27h after addition and a component identified as 7α-hydroxyandrostadienedione V was the major component accumulating. However, a component IV which possessed an absorption maximum at 280nm reached a concentration about 10% of that of the 7α-hydroxyandrostadienedione, 43h after addition of the chenodeoxycholic acid. The transformation was terminated 2.5h later, by which time these two components had decreased in concentration, 7α-hydroxy-androstadienedione to about 30% and IV to about 20% of their respective maximum values. Product IV was recovered in 8.2% of theoretical yield. It exhibited spectral properties very similar to both I and II. The high resolution mass spectrum indicated the molecular formula $C_{19}H_{24}O_4$ while the low resolution mass spectrum featured intense mass fragments of m/z 121, 150, 166 and 195, indicating splitting between the C6-C7-C8 bonds in a manner analogous to I, and a strong $M^+-H_2O$ peak ($\frac{M^+-H_2O}{M^+}$ intensity about 6) indicating the presence of one readily removed hydroxy group. The

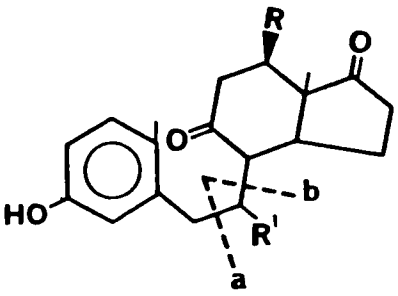
PMR spectrum of IV (Table 1) featured the same aromatic and methyl proton resonances as from I and II and a quartet (J = 7Hz) at δ 3.96, consistent with the resonance from an acyclic C7 proton attached to a hydroxyl bearing carbon and interacting with three nearly-equivalent protons (at C6 and C8). The two proton resonance at δ2.95 which appears as a triplet (J = 7 Hz) is assigned to the benzylic carbon protons at C6. This evidence points to the structure 3,7-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione for IV. The stereochemistry at C8 is assumed to be unchanged from that of chenodeoxycholic acid i.e. 8βH, for the same reasons advanced for the secosteroid II. The configuration at C7 cannot be assigned, for the reasons advanced for II. The name 3,7ζ-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione is proposed for IV.

The biotransformation of hyodeoxycholic acid was carried out in thesame manner as chenodeoxycholic acid and also showed a much lower yield of 280nm-absorbing product than deoxycholic acid. The hyodeoxycholic acid was exhausted 45h after addition and the known catabolite 6α-hydroxy-androstadienedione (6) was at its maximum at this point while a 280nm-absorbing product VI was observed, which reached a maximum value about 2h later. The transformation was terminated 52h after addition of the hyodeoxycholic acid.

Recovery and attempted isolation of the product VI resulted in unexpected difficulties. Silica gel chromatography of the fermentor products with ethyl acetate-hexane containing 0.5% acetic acid effected the separation of the 280nm-absorbing compounds from other products. TLC and HPLC examination, however, showed that the recovered fraction, obtained in about a 12% yield from

hyodeoxycholic acid, now comprised three components in about equal amounts. Further silica gel chromatography of this fraction with ethyl acetate-dichloromethane mixtures effected a nearly complete separation of these compounds from which VI was obtained as fine needles by crystallisation from ethyl acetate.

Compound VI possessed spectral properties similar to those of the other phenolic secosteroids. The high resolution mass spectrum indicated a molecular formula of $C_{19}H_{24}O_4$, as for I and IV. The low resolution mass spectrum featured large fragments at (m/z) 134 (base




I, a → m/z 121, 122

b → m/z 134

R,R' = OH

II, a → m/z 121, 122, 193

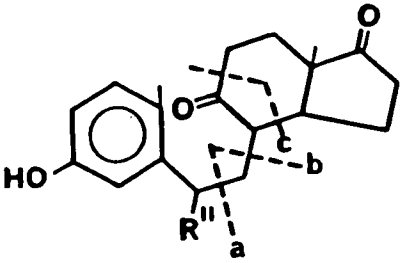
b → m/z 150, 173

R = OH, R' = H

IV, a → m/z 121, 195

b → m/z 150, 166

R = H, R' = OH




VI, VII

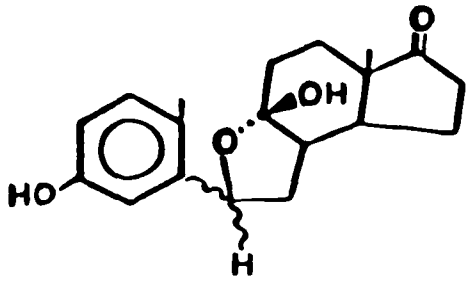
a → m/z 121

b → m/z 134

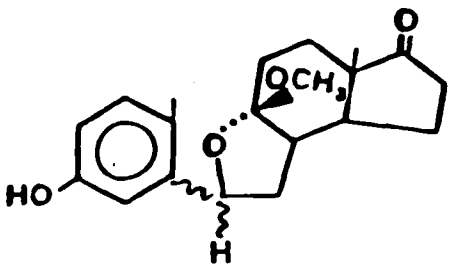
c → m/z 173

VI, R'' = OH, VII, R''

FIGURE 2: Probable mass spectral fragmentation points for the phenolic 9,10-secosteroids I, II, IV, VI and VII which give rise to the major fragments observed.




VI




VII

peak), 121 and 298, with smaller fragments at 159, 173 and 181. The m/z 298 fragment indicates the presence of a readily eliminated hydroxy group ($\frac{M^+-H_2O}{M^+}$ intensity about 3) and the remainder we attribute to the decomposition of a 6-hydroxy-9,10-secosteroid (Fig. 2). In the PMR of VI (Table 1), the resonance at $\delta$ 7.35 was assigned readily to the C4 proton, by comparison with the other secosteroids. The much greater deshielding of this proton than in I, II and IV supported the presence of a hydroxyl at C6. The single proton resonance at $\delta$ 5.32 is assigned to the C6 proton, cf. $\delta$ 3.96 for the corresponding C7 proton in IV, the extra deshielding resulting from the aromatic ring at this benzylic position. The resonance appeared as a quartet, J = 9, 6 Hz, which collapsed to a singlet on irradiation at $\delta$ 1.25, the position expected for the C7 protons to resonate in a 6-hydroxy-9,10-secosteroid structure. However, in such a structure the C7 protons would be free to rotate and equivalent and the C6 proton resonance should appear as a triplet. This apparent non-equivalence of the C7 protons and the ready reactivity of the VI during isolation led to a re-assessment and proposal of a hemi-acetal structure (following an original

suggestion by J.A. Lamberton, personal communication). Support for this structure was found in the IR spectrum, where only one carbonyl absorption was found at 1732 $cm^{-1}$ in solution and 1730 $cm^{-1}$ in a KBr disc (cyclopentanone carbonyl at C17). This demonstrated that no carbonyl group was present at C9, cf. 1708 and 1704 $cm^{-1}$ in I and II, respectively. A stereomodel of the proposed hemi-acetal structure VI shows that one C7-hydrogen bond adopts a position co-planar with the C6-hydrogen bond about the tetrahydrofuran ring and a very small dihedral angle occurs between these bonds, while the other C7-hydrogen bond adopts a dihedral angle of about 130° to the C6-hydrogen bond. Such spatial arrangements are consistent with the observed coupling constants of 9 and 6 Hz for the $\delta$ 5.32 resonance in the PMR spectrum of VI assigned to the C6 proton. The hemi-acetal hydroxyl group at C9 in VI must be in the $\beta$-position relative to the C ring, since stereomodels show that the ether oxygen of the tetrahydrofuran ring can only readily attach at the $\alpha$-position of C9. The stereochemistry of the asymmetric centres in VI are assumed to be the same as those of the parent hyodeoxycholic acid for the same reasons as advanced in discussing the stereochemistry of II above, with the exception of C6 and C9. Some uncertainty occurs as to whether the integrity at the C6 hydrogen remains and in the absence of further evidence the stereochemistry at this position is not specified i.e. 6 $\zeta$. The stereochemistry at the C9 position follows from the discussion above, i.e. 9$\beta$ hydroxyl with an R configuration, unlike the configuration for hyodeoxycholic acid (9S). The structure proposed for VI is therefore 3,9$\beta$-dihydroxy-6$\zeta$,9$\alpha$-epoxy-9,10-seco-1,3,5(10)-androstatrien-17-one.

Compound VII was formed during the chromatography of VI on silica gel, in the presence of acetic acid and was purified by further separation on silica gel. It exhibited spectral properties very similar to VI, including a single carbonyl group absorption (1734 $cm^{-1}$) in the infrared, but with greatly reduced hydroxyl absorption (3380 $cm^{-1}$, very weak) and a high resolution mass spectrum supporting the molecular formula $C_{20}H_{26}O_4$, i.e. VI + $CH_2$. Apart from a different molecular ion of m/z 332, the low resolution spectrum differed from that of VI mainly in the possession of a higher intensity m/z 298 peak (or $M^+-CH_3OH$) and a more intense m/z 121 ion. The PMR spectrum featured a broadened doublet (J = 2.5 Hz) at δ 7.03, cf. 7.35 for VI, which is attributed to a slightly reduced deshielding of the C4 proton, while the C6 proton resonance was very similar to that of VI, i.e. δ 5.35, cf. 5.32 in VI with identical coupling (quartet J = 9,6 Hz), collapsing to a singlet on irradiation at δ 1.25. However, the presence of a three-proton methoxyl singlet at δ 3.33 demonstrated that VII differed from VI in the occurrence of a methoxyl group. The TLC behaviour of VII in showing a higher Rf value than VI in neutral and acidic solvent systems confirms the loss of a hydroxyl group. This evidence indicates that VII probably exists in an acetal structure and, by analogy with VI, possesses stereochemistry similar to that compound. The structure proposed for VII is 3-hydroxy-9β-methoxy,6ζ,9α-epoxy-9,10-seco-1,3,5(10)-androstatrien-17-one (VIIa). The ease with which VI is converted to VII is attributed to the presence of methanol and acidic solvents and the known reactivity of hemi-acetals.

The isolation of these four phenolic secosteroids in fair yield confirms their role as catabolites of the bile acids by Pseudomonas spp. (2,8). The substantial yield from deoxycholic acid and fair yield from the other bile acids are in marked contrast to the experience of others (3-8). The greater yield is attributed to the use of extremely limiting aeration conditions employed here (15). By analogy with the investigation of Dodson and Muir into the degradation of 4-androstene-3,17-dione by a Pseudomonas sp. (13), the formation of a 9,10-secosteroid occurs by 9α-hydroxylation of 1,4-androstadiene-3,17-dione (see Fig. 1) and a subsequent spontaneous retro-aldol reaction. It is hypothesised that such a hydroxylation would be oxygen-dependent and is known to be rate limiting (15,16) whereas the previous catabolic sequences are less dependent on oxygen. Consequently, restriction of aeration would be expected to bring about an accumulation of the immediate precursor, as was observed with cholic acid (15). However, by analogy with previous studies into the degradation of 4-androstene-3,17-dione by Nocardia restrictus (16), hydroxylation at the 4 position might be expected to be the next catabolic step to give a 3,4-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione.

Such a hydroxylation would also be oxygen dependent and a consequence could be that some accumulation of the 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione compounds can occur in the oxygen-depleted environment of the fermentor. No direct evidence has been found to show that the 3,4-dihydroxy-9,10-secosteroids are indeed produced in the catabolism of bile acids by Pseudomonas spp., although highly colored conditions have been found to develop in the

fermentor when the depleted conditions of aeration employed here are maintained (15). Such coloration could be attributed to the production of catechols, such as the 3,4-catecholic-9,10-secosteroids and subsequent reactions of this species (17).

## EXPERIMENTAL

UV spectra were recorded in MeOH on a Hewlett Packard 8450A spectrometer, IR spectra on a Beckman Acculab 8 spectrophotometer, PMR spectra in $CDCl_3$ on a JEOL JNM-PS-100 spectrometer at 100 $MH_Z$, with TMS as internal standard. Low resolution mass spectra was obtained at 70 eV on an AEI MS902S sector instrument. High resolution mass spectra were recorded on a Varian MAT 311A instrument with Spectro-system 100 data system. Melting points were uncorrected. HPLC analyses were performed with either a Varian 8500 or Waters M-45 pump connected to, in series, a Waters U6K injector, a μ Bondapak C18 column, a Varian Varichrom UV-visible or Waters 441 UV detector and a Waters R401 Differential Refractometer. For the analyses of bile acid utilisation, the culture samples were processed through a Waters Sep-pak C18 cartridge and analysed by HPLC (11). For analyses of product formation, the methanol extract prepared for bile acid analysis above was injected (10 μl) into the HPLC system using as solvent 50% aqueous methanol with 0.5% acetic acid, at a flow rate of 0.5 ml/min. The 9,10-secosteroids from cholic acid II, chenodeoxycholic acid IV, hyodeoxycholic acid VI and deoxycholic acid I eluted at 13.8, 16.2, 18.6 and 20.4 mins, respectively.

The microorganisms P.putida ATCC 31752 was maintained as described before. The media used for propagating the P.putida ATCC 31752 was of the same composition as used before (11), except that the appropriate bile acid (1 gram per liter) was used as the carbon source for the fermentor inoculum, instead of cattle bile.

The standard culture volume for fermentor growths was 4 liters and inoculum 400 ml. The media used for the fermentor growths contained, per liter, $K_2HPO_4$, 3.5g; $KH_2PO_4$, 1.5g; $(NH_4)_2SO_4$, 2g; $MgSO_4.7H_2O$, 1g; $CaCl_2.2H_2O$, 100 mg; $FeSO_4.7H_2O$, 5 mg; silicone antifoam, 0.25 ml; the appropriate bile acid, 2 or 3 g as specified and the pH adjusted to 7.0, before sterilising at 121° for 20 mins. Where the cells were grown in the fermentor on glycerol as a carbon source, glycerol (1g per liter of culture) was added before sterilisation to the above mixture, minus the bile acid. In such instances the bile acid, as the sodium salt, was dissolved in 250 ml water at pH 7-7.5 and sterilised separately and added to the fermentor when the glycerol was completely utilised, (indicated by a sharp increase in the dissolved oxygen content of the fermentor culture).

The fermentor growths were carried out in the fermentor described previously (11). The fermentor was operated at 25° and 600 rpm and aeration at 50 to 75 ml per min. Dissolved oxygen levels were monitored with a galvanic-type electrode and cell growth was monitored by measurement of fermentor culture turbidity using a Klett-Summerson colorimeter. Bile acid utilisation and development of UV-absorbing products (240 and 280 nm) were measured as described before (11), as was TLC examination of the products. HPLC examination of product formation was monitored separately, as described above. Recovery of the products was carried out by the technique described previously (11).

3,7ζ,12β-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione from cholic acid (compound II).

8g sodium cholate was the sole carbon source in a fermentor growth with the inoculum of P.putida ATCC 31752. Aeration was at 60 ml per min for 3.5h and then 75 ml per min until the growth was terminated after 17h. TLC examination of the products revealed the presence of 3 catabolites and no cholic acid, which was confirmed by HPLC examination. The steroidal products (2.95g) were recovered by elution from an Amberlite XAD-2 column with methanol, the non-steroidal and acidic (<0.1g) products being washed out with water or 50% aqueous methanol. The fraction containing the steroidal products was chromatographed on Merck silica gel G (150g) with ethyl acetate as developing solvent. From this a fraction (0.76g) was obtained, almost pure in II. Recrystallisation of II, recovered from the ethyl acetate fraction, twice from aqueous methanol yielded colorless needles (0.35g) mp 159.5-160.5°, λ max (MeOH) 217 ($\log_{10}$ ε 4.41) and 280nm ($\log_{10}$ ε 4.04) and λ max (MeOH + $OH^-$) 217, 241 and 300 nm, IR max (KBr) 3410, 1730, 1704, 1612, 1590, 1504, 1361, 1257, 1163, 1122, 1054, 860, 820 $cm^{-1}$, δ (in $CDCl_3$ with DMSO-$d_6$) 6.93 (1H, d, J = 8Hz, 1-H); 6.68 (1H, d J = 3Hz, 4-H); 6.56 (1H, q, J = 3, 8Hz, 2-H); 4.04 (2H, q plus fine splitting, 7-and 12-H); 2.84, (2H, quintet, fine splitting, 6-H); 2.21 (3H, s, 19-Me); 1.07 (3H, s, 18-Me); MW 332.158 ($C_{19}H_{24}O_5$ requires 332.1624); m/z 121 (base peak) ($C_8H_9O$), m/z 314 ($M^+-H_2O$), m/z 296 ($M^+-2H_2O$), m/z 193 ($M^+-H_2O-121$), 182 ($M^+-150$), 150 ($M^+-182$), 122.

3,12β-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (compound I) from deoxycholic acid.

P.putida ATCC 31752 was grown in a 12 liter batch in a fermentor with glycerol as the carbon source for 50h, at which time the growth was in a stationary phase. Sodium deoxycholate (25g) dissolved in water (250 ml) was slowly added to the culture and an aeration rate of 60 ml per min maintained. The growth was terminated 18h later, when TLC and HPLC examination revealed that the deoxycholate was fully utilised and three catabolites were visible, with the major product showing a maximum absorbance in the UV at about 280 nm. The steroidal products (14.04g) were recovered by adsorption on Amberlite XAD-2 and subsequent elution with

methanol. A portion of these products (5.8g) was applied to a column of silica gel G (200g) in ethyl acetate-hexane (1:1) and the column was developed with a mixture of these solvents. From this a fraction (2.88g) was obtained by elution with 70% ethyl acetate in hexane, which was apparently pure I. Recrystallisation of I twice with ethanol provided colorless rhombs, mp 174-5°, undepressed on admixture with authentic 3,12β-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione mp 171-2° and the ultraviolet, infrared and mass spectra of the two compounds were also identical.

3,7ζ-dihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (compound IV) from chenodeoxycholic acid.

P.putida ATCC 31752 cells were grown on glycerol (4g) in a 4 liter scale in the fermentor for 7h and sodium chenodeoxycholate (12g) was then added, an aeration rate of about 40 ml air per min being maintained throughout. The growth was terminated 52h after commencement, when TLC and HPLC examination showed that the chenodeoxycholic acid and other acid catabolites were almost fully utilised. Three steroidal catabolites were seen to be present and one showed maximum absorbance at 280 nm. These products (6.23g) were recovered by adsorption on Amberlite XAD-2 and subsequent elution with methanol. A portion of these products (2.34g) was applied to a column of silica gel G and the column developed with a mixture of ethyl acetate in hexane containing 0.5% acetic acid. From this, a fraction (0.25g) was obtained by elution with 70% ethyl acetate, which was very rich in IV. Recrystallisation of this fraction twice from aqueous methanol provided large colorless rhombs (0.20g), mp 171.5-172.5°, λ max (MeOH) 220 ($\log_{10}$ ε 3.75) and 281 nm ($\log_{10}$ ε 3.31) and λ max ($MeOH+OH^-$) 217 ($\log_{10}$ ε 3.81), 241 ($\log_{10}$ ε 3.82) and 300 nm ($\log_{10}$ ε 3.47). IR max (KBr) 3460, 1725, 1698, 1618, 1582, 1500, 1301, 1262, 1161, 1115, 1049, 850, 822 $cm^{-1}$, δ (in $CDCl_3$) 6.98 (1H, d, J = 7Hz, 1-H); 6.63 (1H, d broadened, J = 2Hz, 4-H); 6.58 (1H, q, J = 7, 2Hz, 2-H); 3.96 (1H, q broadened, J = 7Hz, 7-H); 2.95 (2H, t, J = 7Hz, 6-H); 2.23 (3H, s, 19-Me); 1.06 (3H, s, 18-Me); MW 316.1636 ($C_{19}H_{24}O_4$ requires 316.1674); m/z 121 (base peak), m/z 121 (base peak), m/z 298 ($M^+-H_2O$), m/z 195 ($M^+-121$), m/z 166 ($M^+-150$, m/z 150 ($M^+-166$).

3,9β-dihydroxy-6ζ,9α-epoxy-9,10-seco-1,3,5(10)-androstatrien-17-one (compound VI) from hyodeoxycholic acid.

P.putida ATCC 31752 was grown on glycerol (4 g) as the carbon source in a 4 liter batch in a fermentor for 7h and sodium hyodeoxycholate (12g) was then added, with aeration being maintained at 60 ml per min for 48h and 40 ml per min thereafter. The growth was terminated 59h after commencement when HPLC and TLC examination showed that the hyodeoxycholic acid and its acid catabolites were fully consumed. Four catabolites were seen to be present, one of which (VI) showed a maximum absorbance near 280nm. These products (7.12g) were recovered from Amberlite XAD-2. A portion of these products (4.7g) was chromatographed on Silica Gel G (150g) with

ethyl acetate in hexane containing 0.5% acetic acid as the developing solvent. The 60-70% ethyl acetate fraction (1.65g) comprised mainly VI plus two new products, including VII.

The fraction rich in VI was re-chromatographed on Silica gel G (85g) 230-400 mesh, with ethyl acetate in dichloromethane as developing solvent. A fraction (0.28g) eluted with 40% ethyl acetate was rich in VI. Recrystallisation of this from ethyl acetate afforded colorless needles mp 171-172° (0.15g), λ max (MeOH) 221 ($\log_{10}$ ε 3.64) and 282 nm ($\log_{10}$ ε 3.27), λ max (MeOH+$OH^-$) 218 ($\log_{10}$ ε 3.66) 246 ($\log_{10}$ ε 367) and 300 nm ($\log_{10}$ ε 3.34); IR max (KBr) 3450, 3300, 1732, 1613, 1588, 1500, 1465, 1373, 1255, 1240, 1160, 1103, 1046, 977, 962, 898,818 $cm^{-1}$; δ (in $CDCl_3$) 7.35 (1H, d, J = 2.5 Hz, 4-H); 6.92 (1H, d, J = 8 Hz, 1-H); 6.58 (1H, q, J = 8, 2.5 Hz, 2-H); 5.32 (1H, q, J = 9,6 Hz, 6-H); 2.18 (3H, s, 19-Me); 0.93 (3H, s, 18-Me); MW 316.1721 ($C_{19}H_{24}O_4$ required 316.1674; m/z 134 (base peak) 316 ($M^+$), m/z 298 ($M^+-H_2O$), m/z 173.

A portion (3.7 g) of the crude product from a further growth of P.putida ATCC 31752 on hyodeoxycholic acid was dissolved in methanol (20 ml) and the components separated on a Waters Prep LC System 500 using a single Prep Pak-500/$C_{18}$ cartridge with 60% aqueous methanol as solvent, at a flow rate of 100 ml/min. One fraction containing the last eluting component provided nearly pure VI (0.97 g) on careful evaporation at <40°C. A portion (0.10 g) of this fraction was dissolved in 9:1 chloroform - methanol by heating on a water bath, in an attempt to recrystallise the product, which did not meet with success. TLC examination of the solution on silica gel with chloroform-acetone-methanol (70:25:5) (12) or dichloromethane-methanol (9:1) revealed the presence of two components Rf 0.26 and 0.51 and 0.49 and 0.63 on the respective solvent systems. The fraction of VI from the Waters Prep LC separation dissolved in methanol at room temperature revealed the presence of only one spot, Rf 0.26 and 0.49 on these respective solvent systems. The remainder of this fraction was satisfactorily recrystallised from ethyl acetate to give colorless needles mp 171-172°C, undepressed on admixture with VI from the first growth above.

3-hydroxy-9β-methoxy,6ζ,9α-epoxy-9,10-seco-1,3,5(10)-androstatrien-17-one (compound VII).

Silica gel chromatography of the fraction containing compound VI from the first hyodeoxycholic acid fermentation and using an ethyl acetate-dichloromethane mixture as developing solvent provided a fraction (0.26g) quite rich in VII. This fraction was rechromatographed on another silica gel G column with the same solvent mixture. A fraction from this column (0.15g) eluting with 40% ethyl acetate was almost pure VII. Recrystallisation of this fraction from ethyl acetate and then methanol in dichloromethane provided a small amount (0.021g) of fine colorless needles, dec. 165°C (melting at 180°), λ max (MeOH) 206 219 and 282 nm ($\log_{10}$ ε 3.85, 3.77 and 3.30 respectively); λ max (MeOH+$OH^-$) 217, 243 and 302

nm ($\log_{10}$ ε 4.00, 3.88 and 3.46 respectively); IR max (KBr) 3380, 1732, 1608, 1498, 1462, 1260, 1237, 1200, 1165, 1110, 1080, 1028, 910 $cm^{-1}$; δ (in $CDCl_3$) 7.03 (1H, d, J = 2.5 Hz, 4-H); 6.97 (1H, d, J = 8 Hz, 1-H); 6.62 (1H, q, J = 8, 2.5 Hz, 2-H); 5.35(1H, q, J = 9, 6 Hz, 6-H); 3.33 (3H, s, 6-$OCH_3$); 2.27 (3H, s, 19-$CH_3$); 0.95 (3H, s, 18-$CH_3$); MW 330.194 ($C_{20}H_{26}O_4$ requires 330.194) m/z 134 (base peak), m/z 330 ($M^+$), m/z 298 ($M^+$-$CH_3OH$), m/z 173, m/z 159, m/z 147, m/z 145, m/z 121.

ACKNOWLEDGEMENTS

I thank Messrs. I. Griffiths and B. Arantz for technical assistance, Dr. R.A. Leppik for the provision of one of the secosteroids and Dr. J.A. Lamberton, of the CSIRO Division of Applied Organic Chemistry, Melbourne for helpful suggestions. The low resolution mass spectra were recorded by Mr. G.J. Macfarlane through the courtesy of Dr. R. Evans, University of Queensland and the high resolution mass spectra by Mr. K. Shaw, through the courtesy of Dr. F. Whitfield of the Food Research Laboratory, Sydney of this Division of CSIRO. The PMR spectra were recorded by Miss L.K. Lambert through the courtesy of Dr. C.J. Hawkins, University of Queensland. This investigation was funded in part by the Australian Meat Research Committee.

REFERENCES

1. S. Hayakawa, ADV. LIPD RES. 11, 143 (1973).
2. S. Hayakawa, ZEITSCHR. ALLG. MIKROBIOL. 22, 309 (1982).
3. P.J. Barnes, J.D. Baty, R.F. Bilton and A.N. Mason, TETRAHEDRON 32, 89 (1976).
4. M.E. Tenneson, J.D. Baty, R.F. Bilton and A.N. Mason, BIOCHEM. J. 184, 613 (1979).
5. M.E. Tenneson, J.D. Baty, R.F. Bilton and A.N. Mason, J. STEROID BIOCHEM. 10, 311 (1979).
6. M.E. Tenneson, J.D. Baty, R.F. Bilton and A.N. Mason, J. STEROID BIOCHEM. 11, 1227 (1979).
7. R.F. Bilton, A.N. Mason and M.E. Tenneson, TETRAHEDRON 37, 2509 (1981).
8. R.A. Leppik, TETRAHEDRON 37, 1747 (1981).
9. R.A. Leppik, BIOCHEM. J. 202, 747 (1982).
10. R.A. Leppik, BIOCHEM. J. 210, 829 (1983).
11. R.J. Park, STEROIDS 38, 383 (1981).
12. R.A. Leppik, R.J. Park and M.G. Smith, APPL. ENVIRON. MICROBIOL. 44, 771 (1982).
13. R.M. Dodson, and R.D. Muir, J. AM. CHEM. SOC. 83, 4627 (1961).
14. Mitsubishi Chemical Industries Co. Ltd., JAPAN KOKAI TOKKYO KOHO 80,108,289 (1980). (CHEM. ABSTR. 94, 14024 p, 1981).
15. M.G. Smith and R.J. Park, APPL. ENVIRON. MICROBIOL. 48, 108 (1984).
16. C.J. Sih, S.S. Lee, Y.Y. Tsong and K.C. Wang, J. BIOL. CHEM. 241, 540 (1966).
17. D.T. Gibson, K.G. Wang, C.J. Sih and H. Whitlock Jr., J. BIOL. CHEM. 241, 551 (1966).