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DEGRADATION OF DEOXYCHOLIC ACID BY *PSEUDOMONAS* Sp. NCIB 10590

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Abstract—The microbial degradation of deoxycholic acid **1** by *Pseudomonas* NCIB 10590 has been studied and two major products have been isolated and identified as 12β-hydroxyandrosta-1,4-dien-3,17-dione **2** and 12α-hydroxypregna-1,4-dien-3-one-20-carboxylic acid **9**. Three minor products were isolated and evidence is given for the following structures: 12α-hydroxyandrosta-1,4-dien-3,17-dione **4**, 12β-hydroxyandrosta-4-en-3,17-dione **7** and 12ε, 17ε-dihydroxyandrosta-1,4-dien-3-one **8**.

INTRODUCTION

The microbial transformations of bile acids are implicated in the aetiology of breast[1] and colon cancer[2] and are potentially of importance in the commercial production of physiologically active steroids.[3] In the past 30 years bile acid metabolism has been studied using a wide variety of micro-organisms.[4–16] In the majority of cases the transformations involve oxidation of the secondary OH groups to give the corresponding ketones[4,5,7,8,12,13] and dehydrogenation of ring A to give an αβ unsaturated ketone.[4,6,7,8,14–16] The side chain is usually unchanged or loses two C atoms by β-oxidation,[4,8,9,15,16] but in two instances the side chain was completely removed to yield a 17-ketosteroid.[4,16]

In a recent publication[15] we reported the transformation of deoxycholic acid by *Pseudomonas* NCIB 10590 in a mineral salts medium to give a mixture of acidic and neutral products. The major product of the neutral fraction was assigned the structure **2** but the configuration at the C-12 position was not established. In this paper we present evidence for the structure and configuration of the major components of the neutral and acidic fractions. Three minor components of the neutral fraction have been isolated and their structures are discussed.

RESULTS AND DISCUSSION

Pseudomonas sp. NCIB 10590 grew rapidly on a deoxycholate, mineral salts medium of pH 7·2 at 28°. The course of the fermentation was followed by TLC analysis of ethyl acetate extracts of culture aliquots; the extent of UV absorbance at 244 nm of these extracts indicates the amount of ring A αβ unsaturated ketone present. The absorbance of the acidic material reached a maximum after 14 hr and then slowly decreased, whereas that of the neutral components reached a maximum after 18 hr and then rapidly decreased. A ten litre culture was extracted after 14 hr and separated into neutral and acidic fractions. Separation of the neutral fraction by column chromatography yielded a major component **2** and several minor components. Compound **2** was isolated as a crystalline solid and gave a single spot on TLC analysis and a single peak on GLC analysis. The mass spectrum of **2** (Fig. 1) shows a molecular ion at *m/e* 300 and intense ions at *m/e* 121 and 122, suggesting a steroidal 1,4 dienone structure.[17] The compound formed a mono t-butyldimethylsilyl ether **3** with a characteristic ion at *m/e* 357 (M-57) and a molecular ion at *m/e* 414. A minor constituent of the neutral fraction (**4**) was purified by TLC and on GC/MS analysis gave a mass spectrum (Fig. 2) similar to that of **2**. The mass spectrum of the mono t-butyldimethylsilyl ether of **4** was identical to that produced from **2** except for slight differences in relative ion intensities. Confirmation of the structure of ring A is provided in each case by the IR spectrum (1660, 1618, 1600 cm^{-1}, αβ unsaturated ketone), the UV spectrum (λ_{max} 244 nm, di-β-substituted αβ-unsaturated ketone, double bond exocyclic)[18] and by the PMR spectrum (three vinylic protons in the range 3·05–3·98τ). The IR spectrum of each compound contains a peak at 1740 cm^{-1} (**4**), characteristic of OH groups. On oxidation with Jones' chromic acid reagent,[19] **2** and **4** produce the same trione **10**. Hence the transformation suggested by spectroscopic and chemical evidence is

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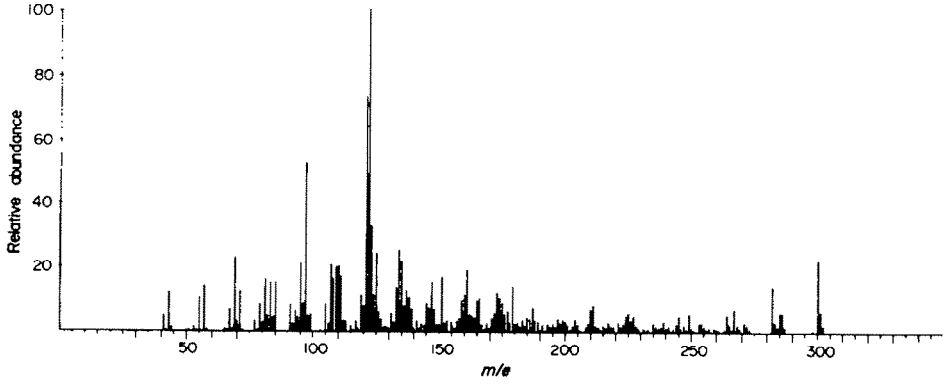
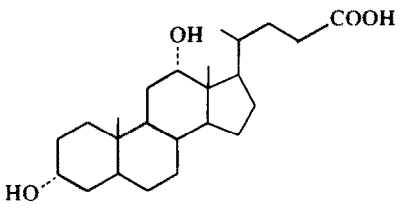


Fig. 1. MS of **2**.



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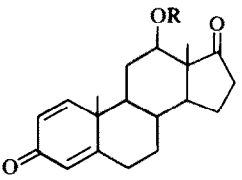

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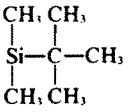
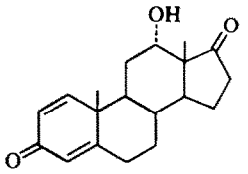
3: R = 



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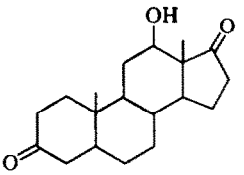

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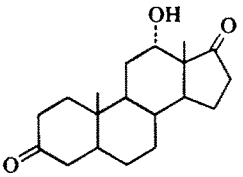

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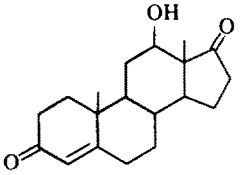

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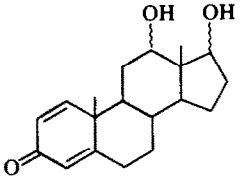

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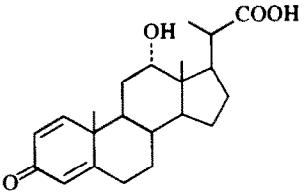

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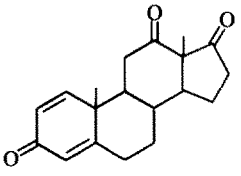

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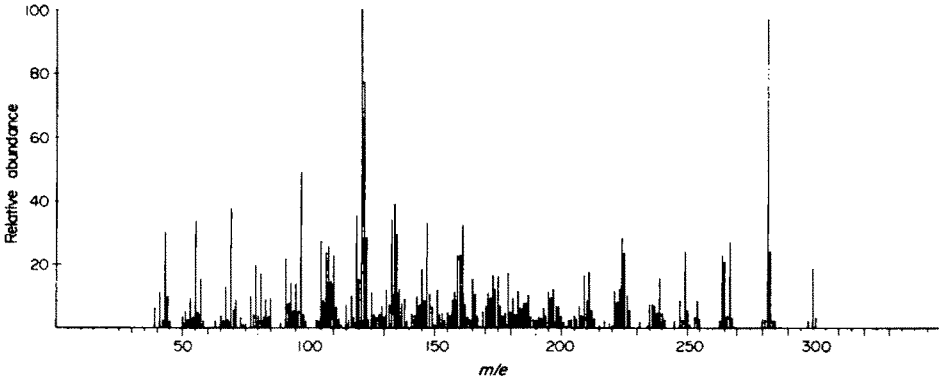

Fig. 2. MS of 4.

oxidative removal of the bile acid side chain to create a ketone group at C_{17}, oxidation of the OH group at C_3 and dehydrogenation at C_1, C_2, C_4 and C_5. Surprisingly, the PMR spectrum indicates that epimerisation has taken place at the twelve position in **2** to produce a 12β OH group. The proton at C_{12} gives rise to a pattern of four lines centred at 6·30τ (1H, four line pattern, J = 5 and 10 Hz) due to spin-spin coupling with the α and β protons at C_{11}. Because the dihedral (Karplus) angles for a 12α proton are about 60° and 180° respectively, two different coupling constants 5 Hz and 10 Hz are observed.[20] In order to check this conclusion the PMR spectrum in the range 5·5–6·5τ of **2** was compared with those of its isomer **4**, methyl deoxycholate and compounds **5** and **6** kindly provided by Prof. G. Spiteller.[21] The results, shown in Table 1, are consistent with the view that a 12α (axial) proton gives rise to a four line pattern, while a 12β (equatorial) proton gives rise to a triplet or broad singlet because it bisects the dihedral angle between the adjacent 11α and 11β protons and only one coupling constant can be discerned. This observation accords with the positions and patterns for protons at C_{12} in 5α steroids.[22] On this basis the OH group of **2** is assigned the 12β configuration and that of **4** the 12α configuration. Further support for this assignment is provided by Spiteller's observation[21] that loss of the elements of water from the molecular ion in the mass spectrometer occurs more readily with the 12α-OH compound **6** than with the 12β-OH compound **5**: in the mass spectrum of **4** there is a pronounced loss of water from *m/e* 300 to give *m/e* 282 as the base peak; this does not occur with **2**. Compound **2** is therefore assigned the structure 12β-*hydroxyandrosta*-1,4-*dien*-3,17-*dione* and **4** the structure 12α-*hydroxyandrosta*-1,4-*dien*-3,17-*dione*. The formation of compound **2** is the first recorded evidence for epimerisation of the 12-OH during the microbial degradation of bile acids under aerobic conditions.

A further minor product **7** of the incubation was observed as a shoulder on the leading edge of the GLC peak from a crude sample of **2**. Several repetitive GC/MS scans were taken over the entire peak. Computer subtraction of the spectra obtained from the trailing edge of the peak (essentially pure **2**) from those of the leading edge (**2** plus **7**) gave the mass spectrum shown in Fig. 3. The molecular ion at *m/e* 302 and the base peak at *m/e* 124 suggest a steroidal 4-en-3-one structure.[17] Oxidation of **7** with Jones' chromic acid reagent gave a compound whose mass spectrum showed a molecular ion at *m/e* 300. Hence, **7** contains one OH group, probably at C_{12} and, as indicated by the relative intensities of the molecular ion and the M-H_2O) ion in the mass spectrum of **7**, probably in the β configuration. Compound **7** is therefore assigned the structure 12β-*hydroxyandrosta*-4-*en*-3,17-*dione*.

Table 1.

	Pertinent spectrum in the range 5·5 – 6·5 τ	
12α-hydroxyandrosta-1,4-dien-3,17-dione (2)	4 line pattern, J, 5, 10 Hz	6·03 τ
12β-hydroxy-5α-androsta-3,17-dione (5)	4 line pattern, J, 5, 10 Hz	6·01 τ
12α-hydroxyandrosta-1,4-dien-3,17-dione (4)	triplet J, 5 Hz	5·86 τ
12α-hydroxy-5α-androsta-3,17-dione (6)	broad singlet	5·87 τ
Methyl 12α-hydroxy-pregna-1,4-dien-3-one-20-carboxylate (methyl ester of 3)	triplet J, 4 Hz	4·04 τ
Methyl deoxycholate (methyl ester of 1)	broad singlet	6·06 τ

A fourth component **8** was isolated by column chromatography of the neutral fraction. It was shown to be a single compound on TLC and GLC analysis. The mass spectrum of this compound is shown in Fig. 4. The ions at *m/e* 266 and *m/e* 284 suggest loss of water from a diol. The base peak at *m/e* 121 indicates the 1,4-dien-3-one structure. Compound **8** could be converted into a di-trimethyl silyl ether, the mass spectrum of which showed a molecular ion at *m/e* 446. Oxidation of **8** with Jones' chromic acid reagent gave the trione (**10**) identical to that obtained from **2** and **4**. Compound **8** is therefore thought to be a diol dienone with the structure 12ξ,17ξ - *dihydroxyandrosta* - 1,4 - *dien* - 3 - *one*.

Crystallisation of the acidic fraction gave a single major component **9**, the methyl ester of which gave a mass spectrum (Fig. 5) showing a low intensity molecular ion at *m/e* 372 and significant ions at *m/e* 354, 267 and 122. The ready loss of water from the molecular ion is a common feature of the mass spectra of the 12α-hydroxy cholanic acid derivatives[23] and the ion at *m/e* 267 corresponds to loss of the side chain from C_{17}. The mass spectrum of **9** is similar to that of the isomer methyl 12α - hydroxypregna - 4,6 - dien - 3 - one - 20 carboxylic acid described by Severina *et al.*[24] The mass spectrum of the latter compound does not show a significant peak at *m/e* 121 or *m/e* 122,

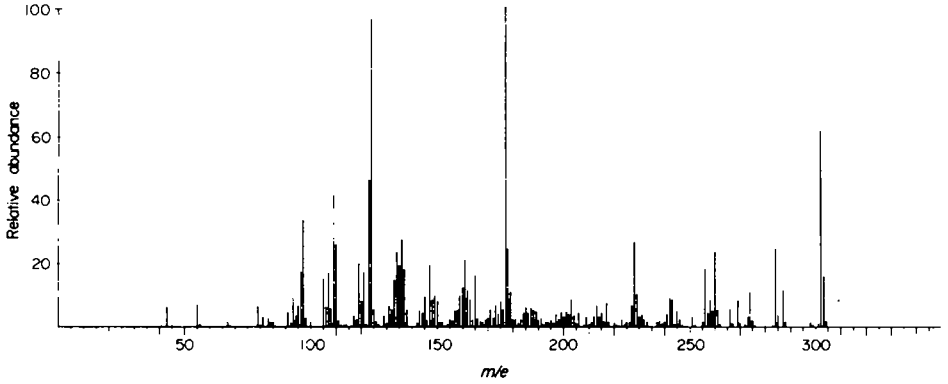

Fig. 3. MS of 7.

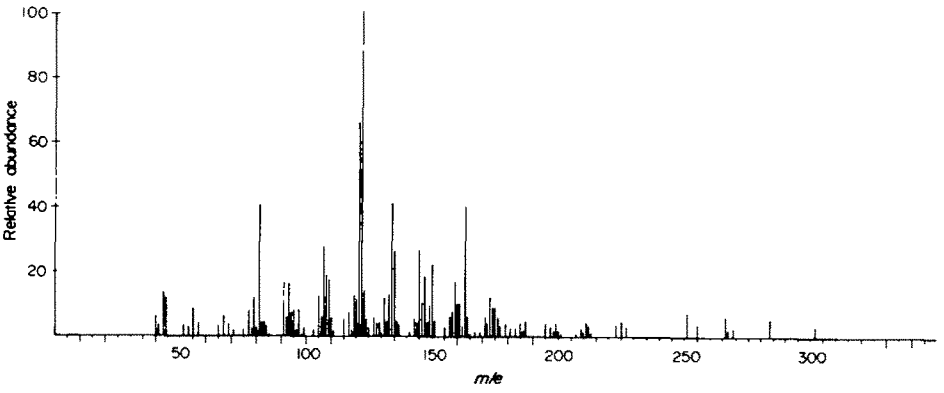

Fig. 4. MS of 8.

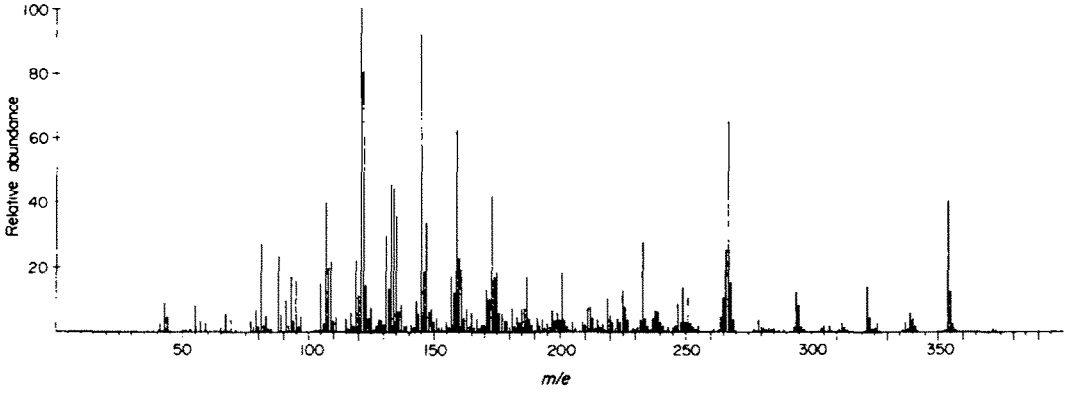

Fig. 5. MS of methyl ester of 9.

but the mass spectrum of **9** shows an ion of high intensity at *m/e* 122, indicating the 1,4-dien-3-one structure, an assignment supported by the UV and PMR spectra. Evidence for the retention of the OH group at C_{12} is provided by two observations: the ready loss of water from the parent ion in the mass spectrum of **9**, and the oxidation of the methyl ester of **9** by Jones' chromic reagent to a ketone, the mass spectrum of which shows an intense molecular ion at *m/e* 370. The PMR spectrum of **9** contains a triplet (J = 3 Hz) at 6·04τ indicating that the OH group retains its α configuration. Compound **9** is therefore assigned the structure 12α - *hydroxypregna* - 1.4 - *dien* - 3 - *one* - 20 - *carboxylic acid.*

Work is in progress to identify other products and elucidate the biochemical pathway of breakdown of deoxycholic acid and to determine the mechanism of epimerisation at C_{12}.

EXPERIMENTAL

UV spectra were recorded in MeOH on a Pye-Unicam S.P. 1800 recording spectrophotometer and IR spectra in KBr discs on a Pye-Unicam S.P. 1200 spectrophotometer. The NMR spectra were obtained in $CDCl_3$ on a Varian HA-100 at 100 MHz. Microanalyses were done by the Butterworth Microanalytical Consultancy Ltd., Teddington.

Separation by GLC was achieved at 250° using 3% OV-1 or 3% OV-17 on 80/100 mesh "Supelcoport" in 5′ × $\frac{1}{8}$″ silanised glass columns. Retention times were measured relative to 5α-cholestane at a flow-rate of 25 cm³ min^{-1} N_2 in a Pye series 104 instrument. GC-MS analyses were carried out using 5′ × $\frac{1}{8}$″ silanised glass columns containing 3% OV-1 or 3% OV-17 on 100/120 mesh Gas-chrom Q. The effluent from the column was passed via a silicone membrane molecular separator into the mass spectrometer (MS-12, Associated Electrical Industries, Manchester, England) operated at 8 kV with a filament current of 300 μA, ionising voltage of 25 eV and source temp. of 230°. The analogue data were analysed by a DS-30 data system.

TLC analysis was performed on 0·25 mm layers of Kieselgel GF_{254} DC-Fertigplatten (E. Merck, Darmstadt) in the solvent system MeOH: dichloromethane, 5:95. UV—absorbing components were detected by observation under light of 254 or 340 nm wavelength and all components were finally visualised by spraying the plate with anisaldehyde reagent and heating at 110° for 10 min.[25]

Purification was achieved by column chromatography on Kieselgel 60,70-230 mesh ASTM (E. Merck, Darmstadt), fractions were eluted with increasing concentrations of MeOH in dichloromethane.

12-Oxosteroids were prepared by treating an acetone soln of the hydroxysteroid with chromic acid reagent at 4°.[17] Acidic steroids were methylated with ethereal diazomethane. Tertiary butyldimethylsilyl ethers were prepared using t-butyldimethylchlorosilane-imidazole (Applied Science Inc.) M.ps were determined on a Koffler hot-stage microscope and were uncorrected. All solvents were redistilled prior to use.

The cells obtained by centrifugation of one litre of *Pseudomonas* NCIB 10590 culture were used to inoculate 10 litres of mineral medium with the composition: K_2HPO_4 0·7 g, KH_2PO_4 0·3 g, KNO_3 1·0 g, $MgSO_4 \cdot 7H_2O$ 0·1 g, sodium deoxycholate 1·0 g, trace element solution 10 cm³, distilled water to 1000 cm³ (final pH 7·2). This culture was incubated at 28° with an aeration of 2 litre min^{-1} and agitation of 400 rev min^{-1}. The course of the transformation was followed by observing the increase in UV absorbance at 244 nm of the steroidal mixture extracted into EtOAc from 2 cm³ samples. When this absorbance reached a maximum after 14 hr incubation the culture was acidified to pH 4 with HCl and the steroids extracted into EtOAc. After drying over $MgSO_4$ the solvent was distilled off at 50° under reduced pressure to yield 3·5 g of a tarry residue which was then dissolved in warm dichloromethane (20 cm³). On cooling a white ppt formed which

was collected by filtration, washed with fresh solvent and found by TLC and GLC to be unchanged deoxycholic acid (500 mg).

Acidic steroids were extracted from the dichloromethane into 6% $NaHCO_3$ solution; addition of HCl to pH 4 followed by EtOAc extraction yielded 1·4 g of a tar. When this was redissolved in EtOAc and left to stand for 3 days at 4° a white ppt formed (1·2 g) consisting of predominantly **9** with traces of a slightly less polar component. This mixture was methylated and the methyl ester of **9** crystallised (657 mg) from $MeOH/CH_2Cl_2$ soln. The less polar component is difficult to separate from **9** and its identity is under investigation.

The dichloromethane soln after extraction of the acids contained 1·6 g of a mixture of four neutral steroids which could not be crystallised. The mixture (1·0 g) was chromatographed on a column of silica gel; combination of the fractions containing **2** and crystallisation from $MeOH/CH_2Cl_2$ gave 600 mg of the pure steroid.

All fractions containing **7** were also contaminated with **2** owing to their similar polarity and the relatively high concentration of the latter component.

The fractions containing **4** were combined and purified further by preparative TLC to yield 15 mg of this steroid.

The most polar fractions contained 80 mg of a steroidal mixture which on preparative TLC yielded 35 mg of crude **8** which could not be further purified by crystallisation.

12β - *Hydroxyandrosta* - 1,4 - *dien* - 3,17 - *dione* (**2**). Recrystallisation of **2** (715 mg) from $MeOH/CH_2Cl_2$ yielded 600 mg **2** m.p. 213–214°. (Found: C, 76·07; H, 8·09. $C_{19}H_{24}O_3$ requires: C, 76·00; H, 8·00%); λ_{max} 244 nm (log ϵ 4·28); IR (KBr disc) 1600, 1618, 1660, 1740, 3495 cm^{-1}; NMR ($CDCl_3$) δ 0·99, 1·23 (6H, s, C_{18} and C_{19} protons), 2·30–2·50 (2H, m, C_{16} protons), 2·96 (1H, s, OH), 3·75 (1H, 4-line m, J = 5 10 Hz, C_{12} proton), 6·02 (1H, s, slight splitting, C_4 proton), 6·18 (1H, d, showing further splitting, J = 10 Hz, C_2 proton), 6·95 (1H, d, J = 10 Hz, C_1 proton). GLC R_f = 0·82 (OV 17).

12α - *Hydroxyandrosta* - 1,4 - *dien* - 3,17 - *dione* (**4**). Recrystallisation of **4** (15 mg) from $MeOH/CH_2Cl_2$ yielded 11 mg. 4 m.p. 221°, λ_{max} 244 nm (log ϵ 4·20); IR (KBr disc) 1600, 1618, 1660, 1740, 3400 cm^{-1}); NMR ($CDCl_3$) δ 0·94, 1·23 (6H, s, C_{18} and C_{19} protons), 2·38–2·45 (2H, m, C_{16} protons), 4·14 (1H, t, J = 3 Hz, C_{12} proton), 4·65 (1H, s, OH), 6·08 (1H, s, slight splitting, C_4 proton), 6·20 (1H, d, showing further splitting, J = 10 Hz, C_2 proton), 6·96 (1H, d, J = 10 Hz, C_1 proton). GLC R_f = 0·60 (OV 17).

Methyl 12α - *hydroxypregna* - 1,4 - *dien* - 3 - *one* - 20 *oate* (*methyl ester of* **9**). Recrystallisation of the methyl ester of **9** (657 mg) from $MeOH/CH_2Cl_2$ yielded 510 mg of the ester, m.p. 237°. (Found: C, 74·29, H, 8·49. $C_{23}H_{32}O_4$ requires: C, 74·20; H, 8·60%); λ_{max} 244 nm (log ϵ 4·23); IR (nujol) 1600, 1620, 1670, 1720, 3450 cm^{-1}; NMR ($CDCl_3$) δ 0·78, 1·22 (6H, s, C_{18} and C_{19} protons), 1·24 (3H, d, J = 6 Hz, C_{21} protons, 3·62 (3H, s, $-OCH_3$ protons), 3·96 (1H, t, J = 3 Hz, C_{12} proton), 6·03 (1H, s, slight splitting, C_4 proton), 6·17 (1H, d, showing further splitting, J = 10 Hz, C_2 proton), 6·97 (1H, d, J = 10 Hz, C_1 proton). GLC R_f = 6·7 (OV 17) (2·4 on 3% OV 1).

Androsta - 1,4 - *diene* - 3,12,17 - *trione* (**10**). Chromic acid (0·65 cm^3, Jones reagent) was added dropwise to a soln containing 500 mg of **2** in acetone (5 cm^3, distilled from $KMnO_4$) and methylene chloride (1 cm^3) at 4°.

The mixture was held at 4° for 16 hr after which time oxidation was shown to be complete by TLC. Excess reagent was decomposed with propan-2-ol (1 cm^3) and the mixture poured into ice-water. The product was isolated in methylene chloride and recrystallised to constant m.p. from methanol-ether to give prisms (400 mg) m.p. 198–200°. (Found: C, 76·78; H, 7·36. $C_{19}H_{22}O_3$ requires: C, 76·50; H, 7·38%); λ_{max} 244 nm (log ϵ 4·17); IR (KBr disc) 1760, 1703, 1682, 1630 cm^{-1}. GLC; R_f = 3·4. (OV 17).

This compound was identical (m.p., GLC, TLC and mass spectrum) with the products of the Jones oxidation of compounds **4** and **8**.

Acknowledgements—We wish to record our grateful thanks to Professor G. Spiteller of the Organic Chemistry Institute, Gottingen University, for the donation of compounds 5 and 6 and to Dr. R. J. Abraham of the Organic Chemistry Department, Liverpool University, for the NMR spectra of compounds 1 and 2 and 4–9.

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