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DEOXYCHOLIC ACID DEGRADATION BY A *PSEUDOMONAS* SP: PHENOLIC AND NEUTRAL PRODUCTS

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Abstract—The microbial degradation of deoxycholic acid by *Pseudomonas* sp. MR108 was studied, and four products were isolated. Evidence is presented that one is the phenol 3,12β - dihydroxy - 9,10 - secoandrosta - 1,3,5(10) - trien - 9,17 - dione (**11**) and that the other three are the neutral compounds 3aαH - 4α - [3' - propionic acid] - 5α - hydroxy - 7aβ - methyl - hexahydro - 1 - indanone - δ - lactone (**12**), 12β - hydroxyandrosta - 1,4 - dien - 3,17 - dione (**10**), and 12α - hydroxyandrosta - 1,4 - dien - 3,17 - dione (**7**).

Research on the microbial degradation of the bile acids has been carried out over the past 25 years. Prominent in this field are Hayakawa *et al.*, who have studied the fermentation of cholic acid (**1a**) by a number of *Streptomyces*, *Corynebacterium* and *Arthrobacter* sp.[1] The products they isolated indicate that these species primarily attack the steroid nucleus of cholic acid, to give intermediates such as **2**, **3** and **4**. Removal of two carbons from the side-chain can also occur, because 23,24-bisnorcholanic acids were also isolated, e.g. **5a**.[1] The latter type of compound has also been isolated by Severina *et al.*[2] from a fermentation of cholic acid by *Mycobacterium mucosum* 1210.

Less work has been done on the microbial fermentation of deoxycholic acid (**1b**). This is partially due to the inability of many of the microorganisms tested to grow on deoxycholic acid as carbon source. Thus, for example, Saburi *et al.*[3] tested eight *Streptomyces* sp. which were capable of growth on cholic acid as carbon source, for growth on other bile acids. All could grow on dehydrocholic acid, five could grow on lithocholic acid, but none could grow on deoxycholic, chenodeoxycholic or hyodeoxycholic acids. Severina *et al.* however, found that *Mycobacterium mucosum* 1210 is capable of growth on both cholic and deoxycholic acids, and, from fermentation of the latter, they isolated the bisnor-acids **5b** and **6**.[4] Compound **6** in particular suggests that, as with cholic acid, attack on the steroid nucleus occurs before removal of the steroid side-chain, because work on the microbial degradation of sterols has shown that 9α - hydroxy - 1,4 - dien - 3 - one steroids are the direct precursors of the 9,10 - seco - steroid intermediates.[5]

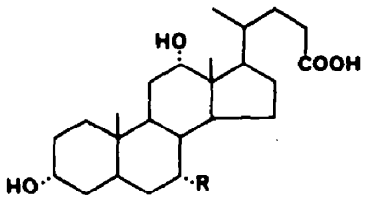
An alternative degradation pathway for bile acids is suggested by the results of Barnes *et al.*[6] From animal faeces, they isolated a *Pseudomonas* sp. which is capable of utilising deoxycholic acid as sole carbon source. From a fermentation of deoxycholic acid by this strain, they isolated the bisnor-acid **8** and the neutral compound **10** as the two main products.[7] A minor product isolated was shown to be **7**, the 12α - hydroxy epimer of **10**. Mass spectral data on two further minor products suggested that they were 12β - hydroxyandrosta - 4 - en - 3,17 - dione **9**, and 12ξ,17ξ - dihydroxyandrosta - 1,4 - dien - 3 - one.[7] These results raise the possibility that *Pseudomonas* sp. are capable of readily removing the side chain of deoxycholic acid completely, and do so before degrading the steroid ring system. This is in contrast to the other species mentioned above. To explore this possibility further, the results obtained with another *Pseudomonas* sp. are described here.

RESULTS AND DISCUSSION

Pseudomonas sp. MR108 was isolated from a soil sample by an enrichment technique,[8] and grew rapidly on a mineral salts medium containing 0.2% deoxycholic acid. For the isolation of degradation products, fermentations were stopped when 85–90% of the substrate had disappeared. Cells were removed by centrifugation, and the cell-free media was pumped through an XAD-2 column to absorb the organic compounds.[9] These compounds were then eluted from the column with aqueous methanol/ammonia. The combined organic extract was separated into acid and non-acid fractions; the acid fraction will be discussed in a subsequent paper.

The non-acid fraction was purified by column chromatography on silica gel with an ethyl acetate/dichloromethane gradient, and progress was monitored by tlc. Two main fractions were obtained, each of which contained essentially one compound. Both were recrystallised to constant m.p., and examination of spectral data suggests that one is 12α - hydroxyandrosta - 1,4 - dien - 3,17 - dione (**7**), and the other 12β - hydroxyandrosta - 1,4 - dien - 3,17 - dione (**10**). This was supported by the good agreement with the published spectral data of Barnes *et al.* for these two compounds.[7] However, the m.p. reported by Barnes *et al.*[7] were 221° for **7** and 213–4° for **10**, whereas the m.p. recorded here were 206.5–7.5 for **7**, and 227–8° for **10**. The reason for these differences is unknown. Never the less, the isolation of these two compounds indicates that *Pseudomonas* sp. MR108, like the strain investigated by Barnes *et al.*[6,7] is capable of removing the side-chain of deoxycholic acid prior to degradation of the steroid nucleus.

An earlier fraction from the above column chromatography was found to contain essentially two compounds, and these were separated by column chromatography on silica gel, using an ethyl acetate/hexane/acetic acid gradient. The two compounds obtained, **11** and **12**, were each recrystallised from ethanol, and each gave one spot only on tlc in two solvent systems. Spectral data suggests that **11** contains a Me alkyl phenol moiety, with H atoms at positions 1, 2 and 4 [IR ($CHCl_3$): 3595 cm^{-1} (OH); 1608, 1586, 1496 cm^{-1} (aromatic ring); λ_{max}^{EtOH} 218 nm (ϵ 7900), 280 nm (ϵ 2700); NMR ($CDCl_3$): 8.34δ (IH, s, Ar–OH, disappears after D_2O exchange), 6.94δ (IH,



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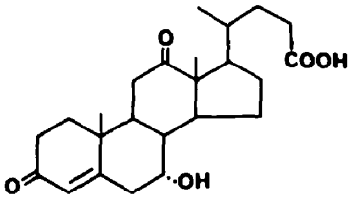
1b R=H



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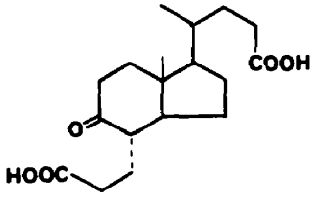

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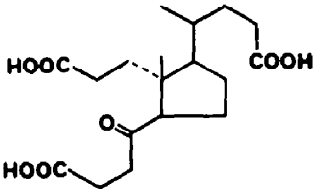

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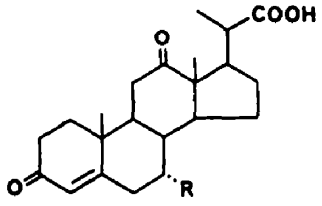
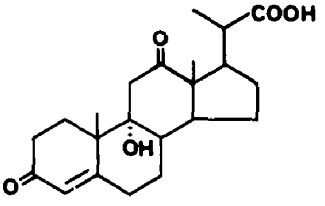


5a R=OH
5b R=H



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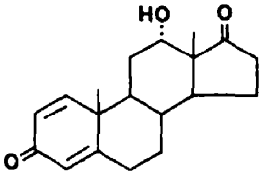

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broad d, C_1-proton, J 7.5 Hz) 6.65δ (IH, skewed d, C_4-proton, J 2 Hz), 6.58δ (IH, d of d, C_2-proton, J 2 and 7.5 Hz), 2.23δ (s, Ar–Me)]. The NMR indicates that one other OH is present [3.58δ (IH, d, J 2 Hz, disappears after D_2O exchange)], with an associated proton which gives a signal at 4.03δ (IH, eight lines, which collapses to four after D_2O exchange). Comparison of the position and band shape of this 4.03δ signal with the data of Bridgeman *et al.*[10] suggests that the OH group is 12β-OH. The IR spectrum indicates that **11** also contains two ketones, one in a 6-membered ring (1708 cm^{-1}), and the other in a 5-membered ring (1728 cm^{-1}). The MS of **11** (Fig. 1) shows a parent ion at *m/e* 316. On this evidence, the most plausible structure for this compound, as a metabolite of deoxycholic acid, is 3,12β - dihydroxy - 9,10 - secoandrosta - 1,3,5(10) - trien - 9,17 - dione (**11**), a compound which has not previously been reported. Such a structure would readily explain the two main peaks in the MS, i.e. the peak at *m/e* 121 from cleavage β to the aromatic ring and the peak at *m/e* 134 from cleavage β to the 9-keto function.

High resolution mass spectral examination of the fourth compound isolated, **12**, showed that its parent ion at *m/e* 222 (MS: Fig. 2) corresponds to $C_{13}H_{18}O_3$ (Found: 222.1257; calc. for $C_{13}H_{18}O_3$: 222.1256). The IR spectrum suggests that **12** contains no OH group (no absorption 4000–3100 cm^{-1}), but could contain both a 5-membered ring ketone and an ester function (1734 cm^{-1}). The latter is supported by the weak absorption in the UV (λ_{max}^{EtOH} 286 nm, ϵ 40). Comparison of the IR spectrum of **12** with that published by Wang *et al.*[11] for 3aαH - 4α - [3' - propionic acid] - 5α - hydroxy - 7aβ - methyl - hexahydro - 1 - indanone - δ - lactone showed that the two spectra are virtually identical, and thus **12** is tentatively identified as being the same compound. This is supported by the good agreement between the m.p. of compound **12** (123–4°) and that reported by Wang *et al.*[11] for the above compound (122–5° after recrystallization, 124–7° after sublimation). Wang *et al.* isolated this compound from a fermentation of androst - 4 - en - 17β - ol - 3 - one by *Nocardia restrictus*, but its isolation from a bile acid fermentation has not been reported to date. It is of interest to note that the 12 - OH group present in deoxycholic acid is no longer present in **12**. A similar dehydroxylation has been reported by Hayakawa to occur once the A and B rings of cholic acid have been removed (as, e.g. in **3**), although in their case, some or all of the side-chain is still present.

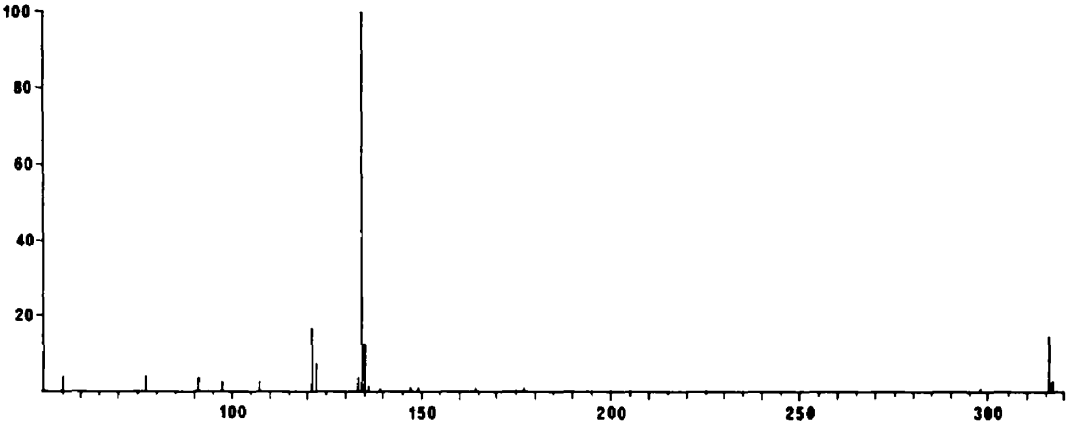

Fig. 1. Mass spectrum of **11**.

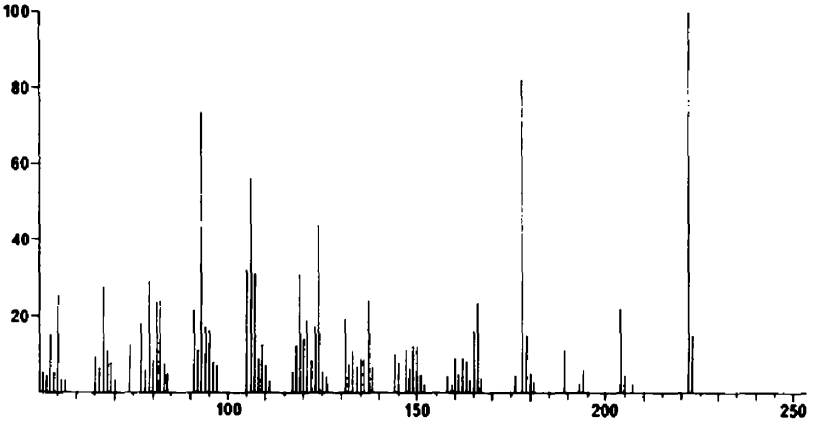

Fig. 2. Mass spectrum of **12**.

Thus, on the present results, *Pseudomonas* spp. do appear to differ from the other microorganisms examined in their mode of degradation of bile acids, in that they remove the side-chain prior to ring degradation. A possible degradation pathway is shown in Fig. 3.

EXPERIMENTAL

UV spectra were recorded in EtOH on a Cary 118 Spectrophotometer, IR spectra in $CHCl_3$ on a Beckman Acculab 8 Spectrophotometer, and NMR spectra in $CDCl_3$ on a Jeol JNM-PS-100 at 100 MHz. MS were obtained on an AEI MS902S Mass Spectrometer, operated with an ionizing voltage of 70 eV.

All solvents were redistilled prior to use. M.p. are uncorrected.

Hplc analyses were performed with a Varian 8500 Pump, fitted with a Waters U6K Injector, a Waters Differential Refractometer, and a Waters μBondapack column. For the analysis of deoxycholic acid utilisation in a fermentation, media (6 ml) was acidified (0.4 ml M HCl) and extracted with EtOAc (1.5 ml). Brief centrifugation was normally needed to break the emulsion. 15 μl of the EtOAc layer was injected, and eluted with 80% aq. MeOH 0.5% HOAc, at 50 ml/hr. The deoxycholic acid eluted at about 12 min.

Tlc analyses were performed on Merck 0.2 mm Kieselgel 60 F 254 plastic tlc sheets, using the two solvent systems EtOAc:CH_2Cl_2 = 60:40 (which separates **7** and **10**) and EtOAc:hexane:AcOH = 60:30:2 (which separates **11** and **12** well). Each plate was developed twice for improved resolution. UV-absorbing compounds were detected by observation under light of 254 nm, and then all components were made visible by spraying the plate with anisaldehyde reagent and heating at 125° for 4 min.[12] After spraying and heating, **7** gave a grey-brown, **10** a yellow-brown, **11** a darkish green, and **12** a lettuce-green, spot.

The media used to grow MR108 contained, per litre: K_2HPO_4 3.5 g, KH_2PO_4 1.5 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 100 mg, NaCl 50 mg, $CaCl_2 \cdot 2H_2O$ 10 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, deoxycholic acid 2.0 g. The pH of the media was adjusted to 7.0 before sterilising. Solid media were prepared using 15 g Difco Bacto agar per litre, and contained, in addition to the above ingredients, Difco Bacto yeast extract 100 mg, but only 1.0 g deoxycholic acid, per litre.

To isolate organisms capable of utilising deoxycholic acid as sole carbon source, an abattoir soil sample (about 0.1 g) was added to the above media (10 ml), supplemented with 0.01% yeast extract, and incubated with shaking for 9d at 30°. Portion was diluted 1:10 with fresh media and, after a further 9d incubation, portion again diluted 1:10 with fresh media. After 7d, a loopful of the 3rd media was plated out onto solid media, and incubated at 30° until growth occurred. Individual colonies were purified by re-plating, and one of these, MR108, selected for the work described here.

For the isolation of degradation products, a 1 l. growth of MR108 in the above media was used to inoculate 15 l. of the same media. The latter was incubated at 30°, with stirring at 500 rpm and aeration at 200 ml min^{-1}, in a VirTis Fermenter (The VirTis Company, Gardiner, New York). Foaming was controlled by the addition of BDH polypropylene glycol 2025. Deoxycholic acid utilisation was followed by hplc and, when 85–90% had been utilised, aeration was stopped and the medium cooled. The cells were removed by centrifugation, and the supernatent pumped through an XAD-2 column (1 kg). The column was washed with water until the eluate was phosphate free (about 2.5 l.). The organic compounds were eluted with 50%, 75% and 100% MeOH in water/0.3 M NH_3. The combined organic extract (about 9.8 g from 15 l.) was partitioned between $CHCl_3$ and 3% Na_2CO_3 aq.

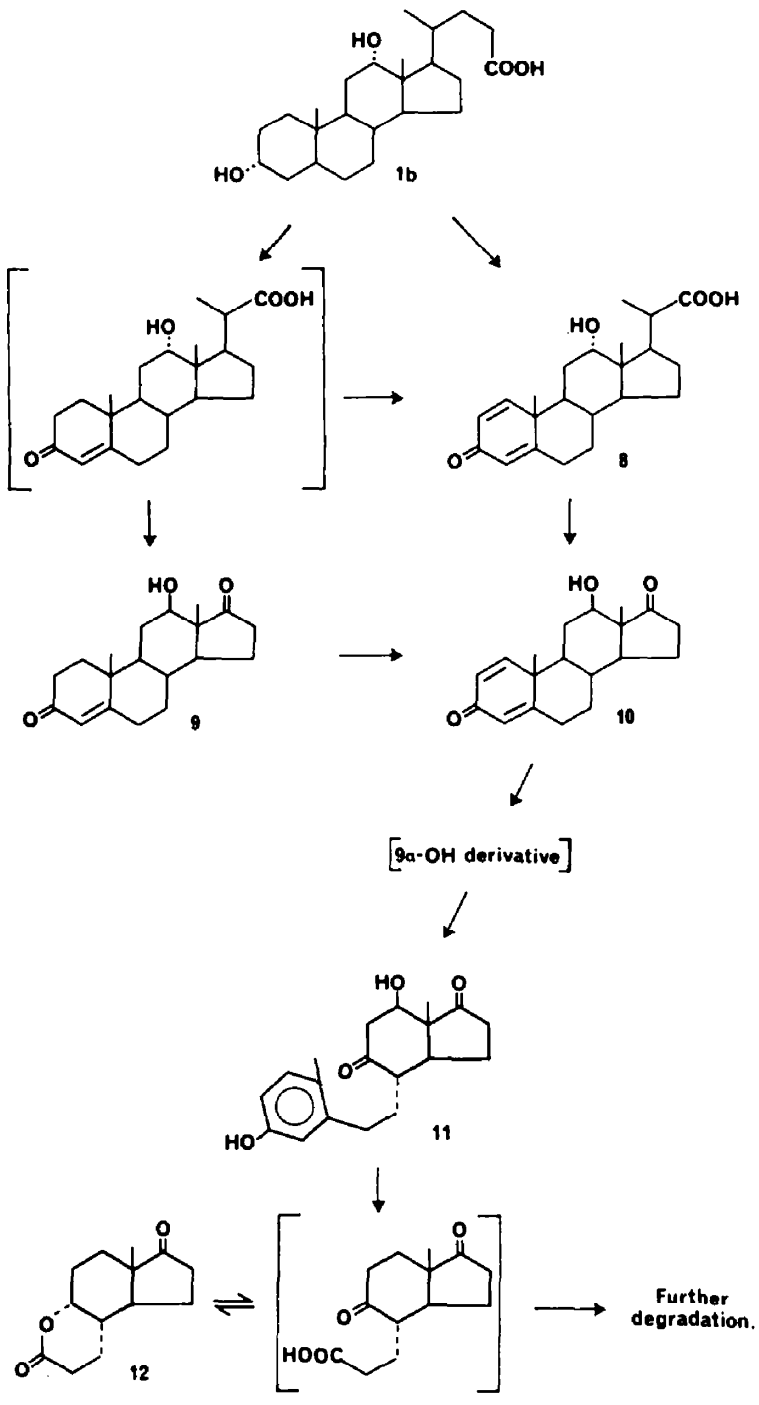

Fig. 3. Possible pathway for the degradation of deoxycholic acid by *Pseudomonas* sp.

The organic phase was evaporated to a residue (1.6 g), and chromatographed on Merck Kieselgel 60, 63–200 μ (50 g), with a 20–100% EtOAc in CH_2Cl_2 gradient. Apart from the fraction (590 mg) which contained the antifoam agent, two main fractions were obtained, one (270 mg) that contained **10** and the other (146 mg) that contained **7**. An earlier fraction (61 mg), that contained **11** and **12**, was rechromatographed on Merck Kieselgel 60, 40–63 μ (22 g), with a 50–70% EtOAc in hexane (all 0.5% HOAc) gradient, to give **11** (29 mg) and **12** (34 mg).

12β - *Hydroxyandrosta* - 1,4 - *dien* - 3,17 - *dione* (**10**). **10** (530 mg) was recrystallised twice from EtOH to yield 330 mg **10**, m.p. 227–8°. λ_{max}^{EtOH} 242 nm (ϵ 15,800); IR ($CHCl_3$) 3570, 1728, 1660, 1620, 1600 cm^{-1}; NMR ($CDCl_3$) 7.03δ (IH, d, J = 10 Hz, C_1 proton), 6.24δ (IH, d of d, J = 2, 10 Hz, C_2 proton), 6.09δ (IH, broad s, C_4 proton), 3.77δ (IH, 8 line m, J = 2 Hz [12αH–12βOH] 4 Hz [12αH–11αH] 10 Hz [12αH–11βH] $C_{12\alpha}$ proton), 3.06δ (IH, d, J = 2 Hz, $C_{12\beta}$OH), 1.27δ (s, C_{19} protons) 1.03δ (s, C_{18} protons); MS parent ion at *m/e* 300.1713, calc. for $C_{19}H_{24}O_3$: 300.1725.

12α - *Hydroxyandrosta* - 1,4 - *dien* - 3,17 - *dione* (**7**). **7** (290 mg) was recrystallised from EtOH to yield 106 mg **7**, m.p. 202–3°. Portion was recrystallised twice from EtOAc, to yield **7**, m.p. 206.5–7.5°. λ_{max}^{EtOH} 243 nm (ϵ 13,600); IR ($CHCl_3$) 3590, 1730, 1660, 1620, 1600 cm^{-1}; NMR ($CDCl_3$) 7.01δ (IH, d, J = 10 Hz, C_1 pro-

ton), 6.23δ (IH, d of d, J = 2, 10 Hz, C_2 proton), 6.10δ (IH, broad s, C_4 proton), 4.17δ (IH, 4 line m [i.e. 6 line pattern, with overlap of lines 2 and 3, and 4 and 5], which collapses to a t after D_2O exchange, J = 3 Hz [12βH-12αOH] 3 Hz [12βH-11α and βH], $C_{12\beta}$ proton), 2.23δ (d, J = 3 Hz, lost after D_2O exchange, $C_{12\alpha}$OH), 1.24δ (s, C_{19} protons), 0.94δ (s, C_{18} protons); MS parent ion at *m/e* 300.1731, calc. for $C_{19}H_{24}O_3$: 300.1725.

3,12β - *dihydroxy* - 9,10 - *secoandrosta* - 1,3,5(10) - *trien* - 9,17 - *dione* (**11**). **11** (61 mg) was recrystallised from EtOH to yield 26 mg **11**, m.p. 169–170°, M^+ 316 λ_{max}^{EtOH} 218 nm (ϵ 7900) 280 nm (ϵ 2700); IR ($CHCl_3$) 3595, 1728, 1708, 1608, 1586, 1496 cm^{-1}; NMR ($CDCl_3$) 8.34δ (IH, s, C_3OH), 6.94δ (IH, broad d, J = 7.5 Hz, C_1 proton), 6.65δ (IH, skewed d, J = 2 Hz, C_4 proton), 6.58δ (IH, d of d, J = 2, 7.5 Hz, C_2 proton), 4.03δ (IH, 8 line m, J = 2 Hz [12αH-12βOH] 6 Hz [12αH-11αH] 11 Hz [12αH-11βH] $C_{12\alpha}$ proton), 3.58δ (IH, d, J = 2 Hz, $C_{12\beta}$OH), 2.23δ (s, C_{19} protons), 1.20δ (s, C_{18} protons); MS parent ion at *m/e* 316.1655, calc. for $C_{19}H_{24}O_4$: 316.1674.

3aα - H - 4α - [3' - *Propionic acid*] - 5α - *hydroxy* - 7aβ - *methyl* - *hexahydro* - 1 - *indanone* - δ - *lactone* (**12**). **12** (57 mg) was recrystallised from EtOH to yield 33 mg **12**, m.p. 123–4° λ_{max}^{EtOH} 286 nm (ϵ 40); IR ($CHCl_3$) 1734 cm^{-1}; NMR ($CDCl_3$) 4.54δ (IH, 4 line m, $C_{5\beta}$ proton) 0.93δ (3H, s, 7aβ-Me), MS parent ion at *m/e* 222.1257, calc. for $C_{13}H_{18}O_3$: 222.1256.

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