Deoxycholic acid degradation by a *Pseudomonas* species

Acidic intermediates from the initial part of the catabolic pathway

Raymond A. LEPPIK

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

(Received 4 November 1981/Accepted 20 November 1981)

The microbial catabolism of deoxycholic acid by a *Pseudomonas* species was studied, and three acidic products were isolated as their methyl esters. Evidence is presented that the compounds are methyl 3 α ,12 α -dihydroxy-23,24-dinor-5 β -cholan-22-oate, methyl 12 α -hydroxy-3-oxo-5 β -cholan-24-oate and methyl 12 α -hydroxy-3-oxo-23,24-dinor-5 β -cholan-22-oate.

Previous studies have indicated that there are at least two main pathways for the microbial catabolism of the bile acids. The first pathway proceeds by primary splitting of the A- and B-rings of the steroid nucleus. This pathway was largely elucidated by Hayakawa and co-workers [see review by Hayakawa (1973)] during studies of the fermentation of cholic acid (Ia) by a number of *Streptomyces* and *Corynebacterium* species. Information on this pathway was also obtained by Severina and co-workers, who examined the fermentation of cholic acid (Ia) (Severina *et al.*, 1968, 1969a) and deoxycholic acid (Ib) (Severina *et al.*, 1969b) by *Mycobacterium mucosum* 1210. These results suggest that, although the five micro-organisms examined each have their own individual variations of the catabolic pathway, a generalized statement for the fate of the A- and B-rings of cholic acid can be formulated as follows: cholic acid (Ia) $\rightarrow$ 7 α -hydroxy-3-one $\rightarrow$ 7 α -hydroxy-4-en-3-one $\rightarrow$ 4,6-dien-3-one $\rightarrow$ 4-en-3-one $\rightarrow$ [9 α -hydroxy-4-en-3-one] $\rightarrow$ [3,4-dihydroxy-9,10-seco-1,3,5(10)trien-9-one] $\rightarrow$ indane acid (II) $\rightarrow$ further degradation (Hayakawa, 1973). The hypothetical 9 α -hydroxy and 9,10-seco compounds were postulated by Hayakawa *et al.* (1967) by analogy with work done on the catabolism of androst-4-ene-3,17-dione. A significant feature of this pathway is that only three of the five micro-organisms examined were found to shorten the bile acid side chain, to form dinor-acid products. Furthermore, none appeared able to completely remove the side chain until the ring system was degraded, as evidenced by the isolation of such compounds as II, III, and IV. Although less work has been done on the catabolism of deoxycholic acid (Ib), the isolation of the dinor-acids V and VI by Severina *et al.* (1969b)

suggests that deoxycholic acid can also be degraded by this pathway; however, as there is no 7 α -hydroxy group present, there would presumably be no 4,6-dien-3-one intermediate in this case. The isolation of compound VI, the only 9 α -hydroxy compound isolated from a bile-acid fermentation, adds support to the above postulated catabolic pathway.

In the second main pathway, the side chain is the primary point of attack, with no ring rupture detected until the side chain is completely removed, as evidenced by the isolation of such compounds as VII and VIII (Leppik, 1981a). This pathway was demonstrated in studies involving *Pseudomonas* species, for the catabolism of such bile acids as cholic acid (Ia) (Tennessee *et al.*, 1979) and deoxycholic acid (Ib) (Barnes *et al.*, 1976; Leppik, 1981a,b). Evidence indicates that this pathway is also used by a *Bacteroides* species (Owen *et al.*, 1977) and an *Escherichia coli* strain (Tennessee *et al.*, 1977). It has been observed that, concurrent with side-chain removal, there is modification of the A-ring, as 4-en-3-one and 1,4-dien-3-one compounds have been isolated (e.g. by Tennessee *et al.*, 1979).

In the present paper the second pathway is explored further, using deoxycholic acid as substrate, and compounds from the first part of the pathway are described.

Results and discussion

Pseudomonas sp. A.T.C.C. 31753 (formerly MR108), when grown on deoxycholic acid as carbon source, produces a range of acidic and non-acidic intermediates. The separation and identification of the main non-acidic compounds has been

(Ia) R=OH

(Ib) R=H

II

III

IV

V

VI

VII

VIII

IX

X

XI

described (Leppik, 1981a). To examine the acidic fraction, the acids were first methylated with diazomethane, then the methyl esters were subjected to column chromatography on silica gel, with a gradient of 20–100% ethyl acetate in dichloromethane. When fractions were examined by t.l.c., on plates that would fluoresce when exposed to 254 nm light, it was found that the main compounds eluted could be considered in three groups. The first group consisted of two compounds that were not u.v.-absorbing and which could be made visible with anisaldehyde reagent. These overlapped somewhat with the second group, which consisted of a number of u.v.-absorbing compounds. The third group to be eluted consisted of further non-u.v.-absorbing compounds. The first and third groups will be discussed in the present paper.

The third group contained two main components, which partially overlapped. The fractions which contained both components were resolved by repeated column chromatography, with a gradient of 50–70% ethyl acetate in dichloromethane. The less polar (and major) component was shown, by t.l.c. and spectral comparisons, to be the methyl ester of the substrate, deoxycholic acid (Ib). The more polar component was further purified by column chromatography on silica gel, with a gradient of 55–75% ethyl acetate in hexane (both containing 0.2% acetic acid), after which it was crystallized with difficulty. Spectral examination suggested that the two hydroxy functions present in the substrate (Ib) were still present [n.m.r.: δ 2.20 (s, 2H, disappears after $^2\text{H}_2\text{O}$ exchange), δ 3.63 (m, 1H), and δ 3.93 (m, 1H) p.p.m., the position and band shape of the latter two signals being consistent with that of βH atoms associated with the 3 α - and 12 α -hydroxy groups respectively]. However, mass-spectral data showed that the compound had only a three-carbon side chain [m/e 378 (M^+), m/e 255 [base peak, $M^+ - 2\text{H}_2\text{O}$ —side chain ($\text{CH}_3\text{CHCO}_2\text{CH}_3$ requires 87)]]]. On the basis of this evidence, the compound is methyl 3 α ,12 α -dihydroxy-23,24-dinor-5 β -cholan-22-oate (IX). Confirmation of the structure (IX) was obtained by spectral comparisons with an authentic sample (R. A. Leppik, unpublished work). Although this compound has been known from synthetic work (the methyl ester and the parent acid being first synthesized by Sawlewicz, 1938), this is the first report of the production of this acid in a fermentation. Its formation shows that, in the second catabolic pathway, side-chain degradation can occur before modification to the bile-acid A-ring. This is in contrast with the first catabolic pathway, where Hayakawa (1973) has stated 'it appears that a 4-en-3-one group is introduced into the cholane nucleus prior to shortening of the side-chain'.

The first group eluted contained two main compounds, together with some compounds over-

lapping from the second group. They were resolved by repeated column chromatography on silica gel, with a gradient of 35–45% ethyl acetate in hexane (both containing 0.2% acetic acid), or a gradient of 1–2% methanol in chloroform. Spectral examination suggested that both compounds contain an oxo ('keto') and a hydroxy function [i.r. (chloroform): less polar compound: 3615 cm^{-1} (OH), 1708 cm^{-1} (CO); more polar compound: 3615 , 1706 cm^{-1} ; n.m.r.: less polar compound: δ 1.70 (s, disappears after $^2\text{H}_2\text{O}$ exchange), δ 4.04 (m, 1H); more polar compound: δ 1.86 (d, disappears after $^2\text{H}_2\text{O}$ exchange), δ 3.97 (m, 1H) p.p.m.; the band shape of the signal near δ 4.0 p.p.m. in each case being consistent with that for the βH associated with a 12 α -hydroxy group]. Mass-spectral data indicated that the less polar compound contained a full five-carbon side chain [m/e 404 (M^+), m/e 271 [base peak, $M^+ - \text{H}_2\text{O}$ —side chain ($\text{CH}_3\text{CHCH}_2\text{CH}_2\text{CO}_2\text{CH}_3$ requires 115)]]], whereas the more polar compound had only a three-carbon side chain [m/e 376 (M^+ , base peak), m/e 271 [51% of M^+ , $M^+ - \text{H}_2\text{O}$ —side chain ($\text{CH}_3\text{CHCO}_2\text{CH}_3$ requires 87)]]]. Thus it would appear that the less polar compound is methyl 12 α -hydroxy-3-oxo-5 β -cholan-24-oate (X), and the more polar compound methyl 12 α -hydroxy-3-oxo-23,24-dinor-5 β -cholan-22-oate (XI). This was confirmed by comparison with authentic samples (R. A. Leppik, unpublished work). Both compounds are known from synthetic work, the less polar C-24 ester (and the parent acid) being first synthesized by Yamasaki & Kyogoku (1935), whereas the more polar dinor ester (but not the parent acid) was first synthesized by Lardon & Reichstein (1944) and Riegel & McIntosh (1944). However, only the less polar C-24 parent acid is known from fermentation studies, being reported as a product of deoxycholic acid fermentation by a *Bacteroides* species under anaerobic conditions (Owen *et al.*, 1977). The isolation of these two compounds, together with the compound isolated from the third group, suggests that micro-organisms using the second catabolic pathway are capable of attack on either end of the bile-acid molecule, without a necessary prior attack on one particular end, to initially produce catabolic intermediates modified in the A-ring, or with shortened side chains, or both.

Experimental

The isolation of *Pseudomonas* sp. A.T.C.C. 31753 (formerly MR108), its fermentation of deoxycholic acid, the isolation of the products and the separation of the acidic fraction were described previously (Leppik, 1981a). Spectra (i.r., n.m.r., mass spectra) were recorded as previously described (Leppik, 1981a). Melting points were deter-

mined in glass capillaries by using an Electrothermal melting-point apparatus and are uncorrected.

T.l.c. analyses were performed on Merck silica-gel t.l.c. sheets (Leppik, 1981a). Each plate was developed twice for improved resolution. To monitor the purification of methyl deoxycholate and its dinor analogue (IX), the main solvent systems used were: ethyl acetate/dichloromethane, 1:1, v/v; ethyl acetate/hexane/acetic acid, 30:20:1, by vol.; benzene/dioxan/acetic acid, 40:10:1, by vol. For the 3-oxo compounds X and XI, the main solvent systems used were: ethyl acetate/hexane/acetic acid, 20:30:1, by vol.; benzene/dioxan/acetic acid, 40:10:1, by vol.; methanol/dichloromethane, 3:97, v/v. After spraying the developed plates with anisaldehyde reagent and heating, methyl deoxycholate (Ib) gave slate-blue (its dinor analogue, IX), sky-blue and pinky-red spots (the two 3-oxo compounds X and XI).

For the separation of the acidic compounds, the acidic fraction (Leppik, 1981a) (about 7.8 g) in cold methanol (50 ml) was first methylated by the slow addition of an excess of diazomethane [dissolved in cold diethyl ether, and prepared as described by Vogel (1956)] to the methanol solution cooled in ice. The reaction mixture was evaporated, then partitioned between chloroform and aq. 3% Na_2CO_3 . The organic phase was evaporated to a residue (6.4 g), which was chromatographed on Merck Kieselgel 60 (63–200 μm particle size; 190 g), with a gradient of 20–100% ethyl acetate in dichloromethane. The components of the first group eluted were resolved by further column chromatography on silica gel (40–63 μm particle size; 50 g), with a gradient of 35–45% ethyl acetate in hexane (both containing 0.2% acetic acid), followed by column chromatography with a gradient of 1–2% methanol in chloroform, to give 570 mg of X, and 190 mg of XI. The components of the third group eluted were resolved by repeated column chromatography on silica gel (40–63 μm particle size; 50 g), with a gradient of 50–70% ethyl acetate in dichloromethane. The major component of the third group was shown to be the expected methyl deoxycholate (1.4 g), by t.l.c. and spectral comparisons. The other component, (IX), was obtained with a yield of 110 mg.

Methyl 12 α -hydroxy-3-oxo-5 β -cholan-24-oate (X)

(X) (570 mg) was recrystallized from 20% ethyl acetate in iso-octane, to give 443 mg of X, m.p. 127.5–8.6°C; same m.p. and mixed m.p. as X prepared by synthesis. I.r._{max.} (chloroform): 3615 (OH), 1720 (shoulder, methyl ester), 1708 (3-oxo) cm^{-1} ; n.m.r. (^2H]chloroform): δ 0.73 (s, C_{18} protons), 1.00 (d, C_{21} protons), 1.02 (s, C_{19} protons), 1.70 (s, $\text{C}_{12\alpha}\text{OH}$), 3.67 (s, 3H, methyl ester), 4.04 (m, 1H, $\text{C}_{12\beta}\text{H}$) p.p.m.; mass spectrum: m/e 404

(M^+), 386 ($M^+ - \text{H}_2\text{O}$), 271 ($M^+ - \text{H}_2\text{O}$ - side chain), 253 ($271 - \text{H}_2\text{O}$), 201 [$271 - \text{C}_4\text{H}_5\text{O}$ (A-ring)].

Methyl 12 α -hydroxy-3-oxo-23,24-dinor-5 β -cholan-22-oate (XI)

XI (190 mg) was recrystallized from methanol, to give 115 mg of XI, m.p. 205.5–7°C, same m.p. and mixed m.p. as XI prepared by synthesis. I.r._{max.} (chloroform): 3615 (OH), 1724 (shoulder, methyl ester), 1706 (3-oxo) cm^{-1} ; n.m.r. (^2H]chloroform): δ 0.74 (s, C_{18} protons), 1.02 (s, C_{19} protons), 1.25 (d, C_{21} protons), 1.86 (d, $\text{C}_{12\alpha}\text{OH}$), 3.65 (s, 3H, methyl ester), 3.97 (m, 1H, $\text{C}_{12\beta}\text{H}$) p.p.m.; mass spectrum: m/e 376 (M^+), 358 ($M^+ - \text{H}_2\text{O}$), 344 ($M^+ - \text{H}_2\text{O} - \text{CH}_3$), 326 ($M^+ - \text{H}_2\text{O} - \text{CH}_3\text{O}$), 316 ($M^+ - \text{CO}_2\text{Me} - \text{H}$), 271 ($M^+ - \text{H}_2\text{O}$ - side chain), 253 ($271 - \text{H}_2\text{O}$), 201 [$271 - \text{C}_4\text{H}_5\text{O}$ (A-ring)].

Methyl 3 α ,12 α -dihydroxy-23,24-dinor-5 β -cholan-22-oate (IX)

IX (110 mg) was crystallized with difficulty from aq. 60% methanol, to give 80 mg of IX, m.p. 75–85°C, unaffected by repeated recrystallization; same m.p. range as IX prepared by synthesis. I.r._{max.} (chloroform): 3610 (OH), 1728 (methyl ester) cm^{-1} ; n.m.r. (^2H]chloroform): δ 0.69 (s, C_{18} protons), 0.92 (s, C_{19} protons), 1.23 (d, C_{21} protons), 2.20 (s, 2H, $\text{C}_{3\alpha}\text{OH}$ and $\text{C}_{12\alpha}\text{OH}$), 3.64 (s, superimposed on a broad peak, 4H, methyl ester and $\text{C}_{3\beta}\text{H}$ respectively), 3.93 (m, 1H, $\text{C}_{12\beta}\text{H}$) p.p.m.; mass spectrum: m/e 378 (M^+), 360 ($M^+ - \text{H}_2\text{O}$), 342 ($M^+ - 2\text{H}_2\text{O}$), 329 ($M^+ - \text{H}_2\text{O} - \text{CH}_3\text{O}$), 327 ($M^+ - 2\text{H}_2\text{O} - \text{CH}_3$), 273 ($M^+ - \text{H}_2\text{O}$ - side chain), 255 ($M^+ - 2\text{H}_2\text{O}$ - side chain).

I thank Dr. R. J. Park for helpful discussions and Dr. R. F. Evans and Mr. G. A. Macfarlane, University of Queensland, for the recording of mass spectra on an ARGC (Australian Research Grants Council) funded MS902S instrument. I also thank Dr. C. J. Hawkins and Miss L. K. Lambert, University of Queensland, for the recording of n.m.r. spectra.

References

- Barnes, P. J., Baty, J. D., Bilton, R. F. & Mason, A. N. (1976) *Tetrahedron* **32**, 89–93
- Hayakawa, S. (1973) *Adv. Lipid Res.* **11**, 143–192
- Hayakawa, S., Kanematsu, Y. & Fujiwara, T. (1967) *Nature (London)* **214**, 520–521
- Lardon, A. & Reichstein, T. (1944) *Helv. Chim. Acta* **27**, 713–726
- Leppik, R. A. (1981a) *Tetrahedron* **37**, 1747–1751
- Leppik, R. A. (1981b) *Proc. Int. Ferment. Symp. 6th, London (Canada)*, 1980 in the press
- Owen, R. W., Bilton, R. F. & Tenneson, M. E. (1977) *Biochem. Soc. Trans.* **5**, 1711–1713
- Riegel, B. & McIntosh, A. V. (1944) *J. Am. Chem. Soc.* **66**, 1099–1103
- Sawlewicz, J. (1938) *Rocz. Chem.* **18**, 250 and 755–761

- Severina, L. O., Torgov, I. V., Skrjabin, G. K., Wulfson, N. S., Zaretskii, V. I. & Papernaja, I. B. (1968) *Tetrahedron* **24**, 2145–2153
- Severina, L. O., Torgov, I. V., Skrjabin, G. K., Wulfson, N. S., Zaretskii, V. I. & Papernaja, I. B. (1969a) *Tetrahedron* **25**, 485–491
- Severina, L. O., Torgov, I. V., Skrjabin, G. K., Zaretskii, V. I., Wulfson, N. S. & Papernaja, I. B. (1969b) *Tetrahedron* **25**, 5617–5622
- Tenneson, M. E., Owen, R. W. & Mason, A. N. (1977) *Biochem. Soc. Trans.* **5**, 1758–1760
- Tenneson, M. E., Baty, J. D., Bilton, R. F. & Mason, A. N. (1979) *Biochem. J.* **184**, 613–618
- Vogel, A. I. (1956) *A Textbook of Practical Organic Chemistry*, 3rd edn., pp. 971–973, Longmans Green, London
- Yamasaki, K. & Kyogoku, K. (1935) *Hoppe-Seyler's Z. Physiol. Chem.* **233**, 29–35