Deoxycholic acid degradation by a *Pseudomonas* sp.

Acidic intermediates with A-ring unsaturation

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The microbial catabolism of deoxycholic acid by a *Pseudomonas* sp. was studied, and six further acidic intermediates were isolated, as their methyl esters. Evidence is presented that the compounds are methyl 12 α -hydroxy-3-oxochol-4-en-24-oate, methyl 12 α -hydroxy-3-oxo-23,24-dinorchol-4-en-22-oate, methyl 12 α -hydroxy-3-oxochola-1,4-dien-24-oate, methyl 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oate, methyl 12 α -hydroxy-3-oxochola-1,4,22 E -trien-24-oate and methyl 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4,17(20)-trien-22-oate. On the basis of these compounds, together with the seven intermediates previously reported, a catabolic pathway is proposed.

The microbial catabolism of bile acids has been examined by a number of workers, and the results obtained led Leppik (1980, 1982) to propose that there are at least two main catabolic pathways. The first pathway was largely elucidated by Hayakawa and co-workers (reviewed in Hayakawa, 1973) from studies of the fermentation of cholic acid (1a) by a number of *Streptomyces* and *Corynebacterium* spp. Further information on this pathway was obtained by Severina and co-workers, who examined the fermentation of cholic acid (1a) (Severina *et al.*, 1968, 1969a) and deoxycholic acid (1b) (Severina *et al.*, 1969b) by *Mycobacterium mucosum* 1210. In this pathway, the main attack is on the bile acid nucleus, with sequential rupture and degradation of the B, A, C and then D rings, as evidenced by the isolation of such compounds as (2) (Hayakawa *et al.*, 1967), (3) and (4) (Hayakawa & Fujiwara, 1977). Three of the five micro-organisms examined were also found to be capable of shortening the bile acid side chain, to form dinor acid products. However, none appeared able to completely remove the side chain until the ring system was completely degraded, as seen in compounds (3) and (4). For those micro-organisms capable of shortening the side chain, Hayakawa (1973) has suggested that the introduction of a 4-en-3-one grouping into the A-ring may be necessary before such shortening can occur.

In the second pathway proposed, the side chain is the main point of attack, with no ring rupture observed until after the side chain is completely removed, as evidenced by the isolation of such compounds as (12), (13), (15) and (17) (Leppik, 1981). This pathway was elucidated from studies

involving *Pseudomonas* spp., for the catabolism of such substrates as cholic acid (1a) (Tenneson *et al.*, 1979), deoxycholic acid (1b) (Barnes *et al.*, 1976; Bilton *et al.*, 1981; Leppik, 1980, 1981, 1982) and cattle bile (Park, 1981). It would also appear to be the pathway used by two *Bacteroides* spp. (Owen *et al.*, 1977) and an *Escherichia coli* strain (Tenneson *et al.*, 1977). Concurrent with side-chain removal, modification of the A-ring is also observed, as 3-one, 4-en-3-one and 1,4-dien-3-one compounds have been isolated. However, unlike the first pathway, such A-ring modifications do not appear to be a necessary prelude to side-chain degradation, as the dinor acid (5) has been isolated (as its methyl ester) from a deoxycholic acid fermentation (Leppik, 1982).

In the present paper, the second pathway is explored further, and six compounds are described. A preliminary report on this work has appeared in the proceedings of a conference (Leppik, 1980).

Results

Pseudomonas sp. A.T.C.C. 31753 (formerly MR108) was grown on deoxycholic acid as sole carbon source, until approx. 90% had been utilized. The medium was extracted, and the extract was separated into an acidic and a non-acidic fraction. The isolation of the main compounds in the non-acidic fraction has been described previously (Leppik, 1981). The acidic fraction was methylated, then subjected to silica-gel column chromatography. When fractions were examined by t.l.c., it was found that the compounds eluted could be

Scheme 1. Proposed pathway for the degradation of deoxycholic acid

divided into three groups. The isolation of the main compounds in the first and third groups to be eluted has been described previously (Leppik, 1982). The fractions in the middle group were seen to contain at least five compounds, which were resolved by

repeated column chromatography on silica gel, with an ethyl acetate gradient in hexane, supplemented with 0.2% acetic acid.

Spectral examination of the first compound (6) to be eluted by the ethyl acetate gradient in hexane

suggested that it contained both a 4-en-3-one function [$\lambda_{\max}$, 240 nm (ϵ 17400); i.r., 1662, 1618 cm^{-1} ; n.m.r., δ 5.73 (s, 1H, $\text{C}_{(4)}\text{H}$) p.p.m.] and a 12 α -hydroxy function [i.r., 3620 cm^{-1} ; n.m.r., δ 1.75 (m, disappears after $^2\text{H}_2\text{O}$ exchange), 4.05 (m, 1H, band shape consistent with that for a βH associated with a 12 α -hydroxy group) p.p.m.]. Mass-spectral data indicated that the compound contained a five-carbon side chain [m/e 402 (M^+), m/e 269 [base peak, $\text{M}^+ - \text{H}_2\text{O}$ - side chain ($\text{CH}_3\text{CHCH}_2\text{CH}_2\text{CO}_2\text{CH}_3$ requires 115)]]; These results are consistent with the compound being methyl 12 α -hydroxy-3-oxochol-4-en-24-oate (6).

The second compound to be eluted (7) also appeared to contain 4-en-3-one and 12 α -hydroxy functions (relevant spectral data similar to above). However, mass-spectral data indicated that the compound had a shortened three-carbon side chain [m/e 374 (M^+), m/e 269 [$\text{M}^+ - \text{H}_2\text{O}$ - side chain ($\text{CH}_3\text{CHCO}_2\text{CH}_3$ requires 87)]]; These results suggest that the compound is methyl 12 α -hydroxy-3-oxo-23,24-dinorchol-4-en-22-oate (7).

Next to be eluted was a mixture of two compounds, which could be separated by column chromatography on silica gel with diethyl ether as eluent. The less polar of the two compounds (8) was found to contain a 1,4-dien-3-one function [$\lambda_{\max}$, 244 nm (ϵ 15600); i.r., 1662, 1620, 1604 cm^{-1} ; n.m.r., δ 7.00 (d, J 10 Hz, 1H, $\text{C}_{(1)}\text{H}$), 6.20 (d of d, J 2, 10 Hz, 1H, $\text{C}_{(2)}\text{H}$), 6.06 (broad s, 1H, $\text{C}_{(4)}\text{H}$) p.p.m.] and a 12 α -hydroxy function [relevant spectral data similar to those of compound (6)]. Mass-spectral data indicated that the compound contained a five-carbon side chain [m/e 400 (M^+), m/e 267 [$\text{M}^+ - \text{H}_2\text{O}$ - side chain (115)]]; Thus the compound would appear to be methyl 12 α -hydroxy-3-oxochol-1,4-dien-24-oate (8).

The more polar compound to be eluted by the diethyl ether (9) was also found to have 1,4-dien-3-one and 12 α -hydroxy functions [relevant spectral data similar to those for compound (8)]. However, it appeared to have a five-carbon side chain that contained a *trans*-double bond conjugated to the carboxylic ester [$\lambda_{\max}$, 214 nm (ϵ 18800); i.r., 1710 cm^{-1} (saturated esters absorb near 1730 cm^{-1}); n.m.r., δ 6.85 (d of d, J 9, 16 Hz, 1H, $\text{C}_{(22)}\text{H}$), 5.74 (d, J 16 Hz, 1H, $\text{C}_{(23)}\text{H}$) p.p.m.; mass spectrum, m/e 398 (M^+), m/e 267 [$\text{M}^+ - \text{H}_2\text{O}$ - side chain (113)]]; These results are consistent with this compound being methyl 12 α -hydroxy-3-oxochol-1,4,22 E -trien-24-oate (9).

Spectral data on the fifth compound to be eluted (10) suggested that it also contained both 1,4-dien-3-one and 12 α -hydroxy functions [relevant spectral data similar to those of compound (8)]. However, it appeared to have a shortened three-carbon side chain [mass spectrum, m/e 372 (M^+), m/e 267 [$\text{M}^+ - \text{H}_2\text{O}$ - side chain (87)]]; These results

suggest that the compound is methyl 12 α -hydroxy-3-oxo-23,24-dinorchol-1,4-dien-22-oate (10).

The final compound to be eluted (11) was found to have a peak in the u.v. spectrum at 238 nm (ϵ 26100). In compound (9) discussed above, the $\alpha\beta$ -unsaturated ester (which contains a disubstituted double bond) results in a u.v. maximum at 214 nm (ϵ 18800). The data of Nielsen (1957) show that the transition from a disubstituted to a tetrasubstituted double bond in an $\alpha\beta$ -unsaturated ester function results in a bathochromic shift of approx. 15 nm. Thus the 238 nm maximum for this final compound is compatible with the u.v. peak being a composite peak, resulting from the presence of both a tetrasubstituted $\alpha\beta$ -unsaturated ester [$\lambda_{\max}$ (calc.) approx. 230 nm] and a 1,4-dien-3-one function ($\lambda_{\max}$, approx. 244 nm, ϵ approx. 16000). Other spectral data provide supporting evidence for the presence of the 1,4-dien-3-one function [i.r., 1662, 1622, 1606 cm^{-1} ; n.m.r., δ 7.00 (d, J 10 Hz, 1H, $\text{C}_{(1)}\text{H}$), 6.21 (d of d, J 2, 10 Hz, 1H, $\text{C}_{(2)}\text{H}$), 6.07 (broad s, 1H, $\text{C}_{(4)}\text{H}$) p.p.m.] and for an $\alpha\beta$ -unsaturated ester function [i.r., 1708 cm^{-1} (saturated esters absorb near 1730 cm^{-1})] in a C_3 side chain [mass spectrum, m/e 370 (M^+)]. The absence of relevant peaks from the n.m.r. spectrum supports the presence of a tetrasubstituted double bond, and its location in the 17(20)-position would explain the very small peak in the mass spectrum at m/e 265 [4% of base peak, $\text{M}^+ - \text{H}_2\text{O}$ - side chain (86)]. Spectral data also show that a 12 α -hydroxy function is present. Thus the evidence suggests that this compound is methyl 12 α -hydroxy-3-oxo-23,24-dinorchol-1,4,17(20)-trien-22-oate (11).

Discussion

Six further acidic intermediates have been isolated, as their methyl esters, from the fermentation of deoxycholic acid by *Pseudomonas* sp. A.T.C.C. 31753, and structures are proposed for them. Two of the compounds isolated were found to contain a 4-en-3-one functionality, but to differ in the length of the side chain. One compound (6) still contained the full C_5 side chain of deoxycholic acid (1b), whereas the other compound (7) had a shortened C_3 side chain. The parent acid of compound (6) has been reported as an intermediate of both the catabolic pathway derived by Hayakawa (Hayakawa & Samuelsson, 1964) and of the second pathway proposed by Leppik (1980). The compound has also been reported by Bilton *et al.* (1981), but the m.p. of the ester they isolated (234–235°C) differed from that reported in the present paper (152–153°C) and elsewhere [e.g. Hayakawa & Samuelsson (1964), 149–151°C; Riegel & McIntosh (1944), 144–145°C]. In the case of the first pathway, it is noteworthy that compound (6) was isolated from a

cholic acid (1a) fermentation, some at least of the micro-organisms using that first path being capable of 7 α -dehydroxylation (Hayakawa, 1973). For organisms using the proposed second pathway, such 7 α -dehydroxylations have not been observed in cholic acid fermentations by *Pseudomonas* sp. (Tenneson *et al.*, 1979), but has been observed with two *Bacteroides* spp. under anaerobic conditions (Owen *et al.*, 1977). Turning to the second compound with the 4-en-3-one structure but with the shortened side chain (7), the parent acid of this compound has only been reported as a catabolite of the proposed second catabolic pathway (Leppik, 1980; Bilton *et al.*, 1981). In this case, there was good agreement between the m.p. of the methyl ester reported by Bilton *et al.* (231–233°C) and that found in the present work (226–228°C).

The remaining four compounds reported in the present paper all contained the 1,4-dien-3-one functionality, but differed in their side chains, two having a saturated side chain, and two having side chains containing unsaturation. In the case of the compounds with saturated side chains, one (8) had the full C₇ side chain, whereas the other (10) had a shortened C₃ side chain. The former compound (8) has been isolated in studies on both the first (Hayakawa & Samuelsson, 1964; again from cholic acid as substrate) and the second (Leppik, 1980; Bilton *et al.*, 1981) pathways of bile acid degradation. However, the m.p. reported by Bilton *et al.* (1981) (242–244°C) differed from that found in the present work (149–150°C) and that reported by Hayakawa & Samuelsson (1964) (149–150°C). The latter compound (10), with the shortened side chain, has only been isolated in studies with the second proposed pathway (Barnes *et al.*, 1976; Leppik, 1980), but in both cases it was the major acidic compound formed. There was good agreement between the m.p. found (245–247°C) and that reported by Barnes *et al.* (1976) (237°C).

The remaining two compounds, those with the 1,4-dien-3-one function but with side-chain unsaturation, again differed in side-chain length. One contained the full C₇ side chain (9), and n.m.r. showed that the double bond was *trans*-substituted. The other compound contained a C₃ side chain, with the double bond in the 17(20)-position (11). The stereochemistry of this double bond could not be determined from the spectral data obtained. Neither of these two compounds has been reported, apart from that in the preliminary communication of this work (Leppik, 1980).

The isolation of these six compounds, together with the compounds previously reported (Leppik, 1981, 1982), enables a catabolic pathway to be postulated, as outlined in Scheme 1. For the conversion of deoxycholic acid (1b) into 12 α -hydroxyandrosta-1,4-diene-3,17-dione (12), the

pathway is best considered in two strands, one for A-ring modifications and the other for side-chain degradation. This is because *Pseudomonas* spp. appear to be capable of acting independently on either end of the bile acid molecule, to produce intermediates that contain various structures from either strand, as seen for compounds (5)–(11). In the left-hand strand are shown the proposed sequence of modifications to the A-ring. Similar sequences have been proposed for A-ring changes in a number of other steroids, e.g. for cholesterol, where the sequence for some micro-organisms is 5-en-3 β -ol $\rightarrow$ 4-en-3-one $\rightarrow$ 1,4-dien-3-one (Martin, 1977). In the case of cholic acid (1a), the ability of such micro-organisms as *Corynebacterium simplex* to carry out 7 α -dehydroxylation has led to the following sequence being proposed: 3 α ,7 α -dihydroxy $\rightarrow$ 7 α -hydroxy-3-one $\rightarrow$ 7 α -hydroxy-4-en-3-one $\rightarrow$ 4,6-dien-3-one $\rightarrow$ 4-en-3-one $\rightarrow$ 1,4-dien-3-one (Hayakawa, 1973). For *Pseudomonas* spp., however, the compounds isolated by Tenneson *et al.* (1979) suggest that, for cholic acid as substrate, *Pseudomonas* spp. follow the left-hand strand of Scheme 1, rather than the above sequence from cholic acid, as 7 α -hydroxyandrosta compounds were isolated, but no 4-en-3-one or 1,4-dien-3-one compounds lacking a 7 α -hydroxy group.

In the right-hand strand of the pathway in Scheme 1, side-chain degradation is shown as occurring via a mechanism analogous to that for β -oxidation of fatty acids, to yield acetate (C₍₂₃₎ + C₍₂₄₎) and then propionate (C₍₂₀₎ + C₍₂₁₎ + C₍₂₂₎). Good evidence in support of such a sequence has been obtained by Sih and his collaborators, who showed that fermentation of [24-¹⁴C]lithocholic acid by *Nocardia restrictus* resulted in the formation of acetic acid labelled in the carboxylic acid group (Sih *et al.*, 1967b), and fermentation of the dinor acid 3-hydroxy-23,24-dinor[22-¹⁴C]chol-17(20)-en-22-oic acid by *Nocardia* sp. A.T.C.C. 19170 resulted in the formation of propionic acid, also labelled in the carboxylic acid group (Sih *et al.*, 1967a). The isolation of the $\alpha\beta$ -unsaturated esters (9) and (11) provides further support for such a degradative sequence. The evidence that the double bond in the side chain of compound (9) is *trans*-substituted is significant, as the double-bond intermediates in the β -oxidation of fatty acids are also *trans* (Wakil, 1970). By analogy with the fatty acids, it might be of interest to determine whether CoA derivatives of the bile acids are the true intermediates of the pathway.

For the subsequent degradation of the 12 α -hydroxyandrosta-1,4-diene-3,17-dione (12), it is proposed that epimerization of the 12 α -hydroxy group, to form 12 β -hydroxyandrosta-1,4-diene-3,17-dione (13), is a necessary prelude to 9 α -hydroxylation, possibly to avoid steric hindrance of that hydroxylation reaction. This is an alternative hypo-

thesis to that proposed by Bilton *et al.* (1977), who argue that the 12 α -hydroxy group sterically hinders the removal of the side chain, and that this is relieved by epimerization. The former hypothesis is considered to be the more likely, partly because the results obtained by Bilton *et al.* (1977) are open to more than one interpretation, and partly because no intermediate bile acids containing a 12 β -hydroxy group have been reported, whereas androstane compounds containing a 12 α -hydroxy group [e.g. compound (12)] have been reported by a number of workers (e.g. Barnes *et al.*, 1976; Leppik, 1981; Park, 1981). In the alternative pathway elucidated by Hayakawa (1973), the steric hindrance by the 12 α -hydroxy group would appear to be avoided by oxidation, as the compounds isolated suggest that the 12 α -hydroxy group is oxidized to a 12-oxo function before ring rupture occurs (Hayakawa, 1973).

The 12 β -hydroxyandrosta-1,4-diene-3,17-dione (13) so formed is postulated to be converted into the 9 α -hydroxy intermediate (14) (which may be unstable under growth conditions), followed by the splitting of the 9,10-bond, to give the isolated secophenol (15) (Leppik, 1981). For those micro-organisms using this second pathway, the compounds isolated suggest that the double bond in the 1,2-position is inserted before 9 α -hydroxylation occurs. In the alternative pathway elucidated by Hayakawa (1973), this order does not appear to be obligatory, as both 1,4-dien-3-one compounds (Hayakawa, 1973) and a 9 α -hydroxy-4-en-3-one compound (Severina *et al.*, 1969*b*) have been isolated.

The aromatic ring of the secophenol (15) is then degraded, to give the postulated indane acid intermediate (16), of which the isolated indane lactone (17) would be a by-product (Leppik, 1981). It is noteworthy that the 12-hydroxy group present in deoxycholic acid is no longer present in the indane lactone (17). A similar dehydroxylation was reported by Hayakawa *et al.* (1967), who isolated (2) from a cholic acid (1a) fermentation. These indane compounds are utilized, as they do not remain in the medium when a deoxycholic acid fermentation is allowed to go to completion (R. A. Leppik, unpublished work). The details of the pathway after the secophenol (15) are possibly similar to that proposed for the alternative pathway (Hayakawa, 1973), although in the latter case the ring compounds would still have part or all of the side chain attached.

The pathway proposed here would appear to be generally applicable to *Pseudomonas* spp., on the basis of the results published to date. However, there are some individual strain variations. Thus, for *Pseudomonas* sp. A.T.C.C. 31753, growth under conditions of limited aeration results in the

accumulation of high concentrations of androsta-1,4-diene-3,17-dione intermediates, such as compound (13) from deoxycholic acid (Park, 1981; Leppik *et al.*, 1982). Again, for the other *Pseudomonas* sp. studied in detail, *Pseudomonas* sp. N.C.I.B. 10590, there also are variations. Thus it is capable of forming acidic intermediates in which the 12 α -hydroxy group has been oxidized to an oxo function, and of forming androstane intermediates that contain a 17 β -hydroxy function rather than a 17-oxo function (Bilton *et al.*, 1981). However, such variations are compatible with the pathway proposed in the present paper.

Experimental

U.v. spectra were recorded in ethanol, i.r. spectra in chloroform, and n.m.r. spectra in [²H]chloroform at 100 MHz. Mass spectra were determined with an ionizing voltage of 70 eV. Melting-point determinations and t.l.c. analyses were performed as described previously (Leppik, 1982). The main solvent systems used for t.l.c. were ethyl acetate/hexane/acetic acid (25:25:1, by vol.), toluene/dioxan/acetic acid (40:10:1, by vol.) and, for the separation of compounds (8) and (9), diethyl ether. After spraying the developed plates with anisaldehyde reagent and heating, the 4-en-3-one esters gave brown spots, and the 1,4-dien-3-one esters gave blue spots of various shades.

The isolation of *Pseudomonas* sp. A.T.C.C. 31753, its fermentation of deoxycholic acid (0.2% in minimal medium), the isolation of the products from a 15-litre fermentation and the separation of the acidic fraction were described previously (Leppik, 1981). The methylation of the acidic fraction, and the subsequent separation, by column chromatography, of the methyl esters into the three main groups has been described previously (Leppik, 1982). The methyl esters present in the middle group were separated by repeated column chromatography on silica gel (Merck Kieselgel 60, 40–63 μ m particle size, 50 g per column), with a gradient of 40–55% (v/v) ethyl acetate in hexane (both containing 0.2% acetic acid). For the separation of compounds (8) and (9) on silica gel, it was necessary to use diethyl ether as eluent. As most of the compounds had only limited solubility in ethyl acetate/hexane, they were normally dissolved in dichloromethane, diluted with 0.5 vol. of hexane, and the resultant solution was applied to the column, which was then developed with the ethyl acetate gradient in hexane. The compounds are listed below in order of elution.

Methyl 12 α -hydroxy-3-oxochol-4-en-24-oate (6)

Compound (6) (420 mg) was recrystallized from aq. 70% ethanol, to give 320 mg of compound (6),

m.p. 151–152°C [literature m.p. 149–151°C (Hayakawa & Samuelsson, 1964)], with the following properties: $\lambda_{\max}$, 240 nm (ϵ 17400); i.r._{max}, 3620 (OH), 1730 (C=O, ester), 1622 (C=O, 4-en-3-one), 1618 (C=C, 4-en-3-one), 1176 (C–O, C₍₂₄₎ methyl ester) cm⁻¹; n.m.r., δ 0.75 (s, C₍₁₈₎ protons), 0.99 (d, C₍₂₁₎ protons), 1.18 (s, C₍₁₉₎ protons), 1.75 (C_(12a)OH), 3.67 (s, 3H, methyl ester), 4.05 (m, 1H, C_(12b)H), 5.73 (s, 1H, C₍₄₎H) p.p.m.; mass spectrum, m/e 402 (M⁺), 384 (M⁺–H₂O), 371 (M⁺–OCH₃), 342 (M⁺–H₂O–CH₂CO), 269 (base peak, M⁺–H₂O–side chain), 124 (4-en-3-one) (Found: C, 75.0; H, 9.3; C₂₅H₃₈O₄ requires C, 74.6; H, 9.5%).

Methyl 12 α -hydroxy-3-oxo-23,24-dinorchol-4-en-22-oate (7)

Compound (7) (290 mg) was recrystallized from 35% ethanol in ethyl acetate, to give 180 mg of compound (7), m.p. 226–228°C [literature m.p. 231–233°C (Bilton *et al.*, 1981)], with the following properties: $\lambda_{\max}$, 240 nm (ϵ 17400); i.r._{max}, 3615 (OH), 1728 (C=O, ester), 1662 (C=O, 4-en-3-one), 1616 (C=C, 4-en-3-one), 1164 (C–O, C₍₂₂₎ methyl ester) cm⁻¹; n.m.r., δ 0.75 (s, C₍₁₈₎ protons), 1.17 (s, C₍₁₉₎ protons), 1.24 (d, C₍₂₁₎ protons), 1.91 (s, C_(12a)OH), 3.64 (s, 3H, methyl ester), 3.99 (m, 1H, C_(12b)H), 5.73 (s, 1H, C₍₄₎H) p.p.m.; mass spectrum, m/e 374 (M⁺), 356 (M⁺–H₂O), 314 (M⁺–H₂O–CH₂CO), 269 (M⁺–H₂O–side chain), 124 (base peak, 4-en-3-one) (Found: C, 74.0; H, 9.3; C₂₃H₃₄O₄ requires C, 73.8; H, 9.2%).

Methyl 12 α -hydroxy-3-oxochola-1,4-dien-24-oate (8)

Compound (8) (180 mg) was recrystallized from di-isopropyl ether, to give 110 mg of compound (8), m.p. 149–150°C [literature m.p. 149–150°C (Hayakawa & Samuelsson, 1964)], with the following properties: $\lambda_{\max}$, 244 nm (ϵ 15600); i.r._{max}, 3620 (OH), 1732 (C=O, ester), 1662 (C=O, 1,4-dien-3-one), 1620, 1604 (C=C, 1,4-dien-3-one), 1176 (C–O, C₍₂₄₎ methyl ester) cm⁻¹; n.m.r., δ 0.77 (s, C₍₁₈₎ protons), 0.99 (d, C₍₂₁₎ protons), 1.23 (s, C₍₁₉₎ protons), 1.74 (C_(12a)OH), 3.67 (s, 3H, methyl ester), 4.06 (m, 1H, C_(12b)H), 6.06 (broad s, 1H, C₍₄₎H), 6.20 (d of d, J 2, 10 Hz, 1H, C₍₂₎H), 7.00 (d, J 10 Hz, 1H, C₍₁₎H) p.p.m.; mass spectrum, m/e 400 (M⁺), 382 (M⁺–H₂O), 267 (M⁺–H₂O–side chain), 121 (base peak, 1,4-dien-3-one) (Found: C, 75.0; H, 9.3; C₂₅H₃₆O₄ requires C, 75.0; H, 9.1%).

Methyl 12 α -hydroxy-3-oxochola-1,4,22E-trien-24-oate (9)

Compound (9) (140 mg) was recrystallized from 20% ethyl acetate in di-isopropyl ether, to give 50 mg of compound (9), m.p. contracted and melted at 100°C, with the following properties: $\lambda_{\max}$,

214 nm (ϵ 18800), 242 nm (16200); i.r._{max}, 3620 (OH), 1710 (C=O, $\alpha\beta$ -unsaturated ester), 1662 (C=O, 1,4-dien-3-one), 1620, 1604 (C=C, 1,4-dien-3-one), 1156 (C–O, $\alpha\beta$ -unsaturated C₂₄ methyl ester) cm⁻¹; n.m.r., δ 0.82 (s, C₍₁₈₎ protons), 1.15 (d, C₍₂₁₎ protons), 1.23 (s, C₍₁₉₎ protons), 1.72 (C_(12a)OH), 3.73 (s, 3H, methyl ester), 4.06 (m, 1H, C_(12b)H), 5.74 (d, J 16 Hz, 1H, C₍₂₃₎H), 6.06 (broad s, 1H, C₍₄₎H), 6.20 (d of d, J 2, 10 Hz, 1H, C₍₂₎H), 6.85 (d of d, J 9, 16 Hz, 1H, C₍₂₂₎H), 7.00 (d, J 10 Hz, 1H, C₍₁₎H) p.p.m.; mass spectrum, m/e 398 (M⁺), 380 (M⁺–H₂O), 267 (M⁺–H₂O–side chain), 121 (1,4-dien-3-one) (Found: C, 75.4; H, 8.6; C₂₅H₃₄O₄ requires C, 75.3; H, 8.6%).

Methyl 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oate (10)

Compound (10) (1540 mg) was recrystallized from ethanol, to give 1040 mg of compound (10), m.p. 245–247°C [literature m.p. 237°C (Barnes *et al.*, 1976)], with the following properties: $\lambda_{\max}$, 245 nm (ϵ 16300); i.r._{max}, 3615 (OH), 1724 (C=O, ester), 1658 (C=O, 1,4-dien-3-one), 1618, 1600 (C=C, 1,4-dien-3-one), 1162 (C–O, C₍₂₂₎ methyl ester) cm⁻¹; n.m.r., δ 0.76 (s, C₍₁₈₎ protons), 1.20 (s, C₍₁₉₎ protons), 1.26 (d, C₍₂₁₎ protons), 2.58 (s, C_(12a)OH), 3.63 (s, 3H, methyl ester), 3.98 (m, 1H, C_(12b)H), 6.08 (broad s, 1H, C₍₄₎H), 6.20 (d of d, J 2, 10 Hz, 1H, C₍₂₎H), 7.01 (d, J 10 Hz, 1H, C₍₁₎H) p.p.m.; mass spectrum, m/e 372 (M⁺), 354 (base peak, M⁺–H₂O), 322 (M⁺–H₂O–CH₃OH), 267 (M⁺–H₂O–side chain), 122, 121 (1,4-dien-3-one) (Found: C, 74.4; H, 8.8; C₂₃H₃₂O₄ requires C, 74.2; H, 8.7%).

Methyl 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4,17(20)-trien-22-oate (11)

Compound (11) (180 mg) was recrystallized from ethyl acetate, to give 130 mg of compound (11), m.p. 181–182°C, with the following properties: $\lambda_{\max}$, 238 nm (ϵ 26100); i.r._{max}, 3590 (OH), 1708 (C=O, $\alpha\beta$ -unsaturated ester), 1662 (C=O, 1,4-dien-3-one), 1622, 1606 (C=C, 1,4-dien-3-one), 1146 (C–O, $\alpha\beta$ -unsaturated C₍₂₂₎ methyl ester) cm⁻¹; n.m.r., δ 1.03 (s, C₍₁₈₎ protons), 1.24 (s, C₍₁₉₎ protons), 1.86 (s, C_(12a)OH), 2.02 (t, J 2 Hz, C₍₂₁₎ protons), 3.71 (s, 3H, methyl ester), 4.54 (m, 1H, C_(12b)H), 6.07 (broad s, 1H, C₍₄₎H), 6.21 (d of d, J 2, 10 Hz, 1H, C₍₂₎H), 7.00 (d, J 10 Hz, 1H, C₍₁₎H) p.p.m.; mass spectrum, m/e 370 (M⁺), 352 (base peak, M⁺–H₂O), 338 (M⁺–CH₃OH), 320 (M⁺–H₂O–CH₃OH), 121 (1,4-dien-3-one) (Found: C, 74.6; H, 8.3; C₂₃H₃₀O₄ requires C, 74.6; H, 8.2%).

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