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TRANSFORMATION OF CHOLIC ACID BY THE CULTURE MYCOBACTERIUM N 1210

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Abstract—The microbiological transformation of cholic acid by the culture Mycobacterium N 1210 has been studied. The following products have been isolated in the form of methyl esters: 7α-hydroxy-3,12-diketo-Δ^4-cholenic (IIIa), 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic (IVa), 7α-hydroxy-3,12-diketo-$\Delta^{4,8(9)}$-bisnorcholadienic (Va), 7-hydroxy-3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic (VIa), 12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienic (VIIa), 12α-hydroxy-3-keto-$\Delta^{4,6}$-bisnorcholadienic (VIIIa) and 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic (IXa) acids.

THE progress in the microbiological transformation of steroids in the androstane and pregnane series led different groups of scientists to undertake similar investigations in the field of bile acids, aiming at the preparation of intermediates for the synthesis of steroid hormones and other physiologically active compounds.

Cholic acid, the most available and cheapest bile acid, became the main object for such investigations.

In several publications,[1–11] it has been shown that some cultures of actinomycetes, mycobacteria and fungi are capable of transforming cholic acid into different products when this acid is the only source of carbon. As a rule the transformation includes oxidation of OH groups to keto-groups, Δ^4-dehydrogenation in ring A and sometimes β-oxidation of the side chain (for actinomycetes and fungi). No splitting of the bile acid side-chain was observed for mycobacteria. Usually the process of transformation required from 7–10 days to 2 months and the yield of the final products was very low.[4, 5, 9, 10]

It has been reported[12] that from different samples of soil, 113 cultures of microorganisms are capable of utilising cholic acid as the only source of carbon. Most of these cultures were shown to form the Δ^4-3-keto-grouping in the ring A of cholic acid but one of them—Mycobacterium N 1210—brings about the complete conversion of cholic acid in 70 hr of fermentation. In this paper we describe the results of the transformation of cholic acid by this culture.

RESULTS AND DISCUSSION

After transformation of cholic acid by the culture Mycobacterium N 1210 (the absence of cholic acid in the cultural medium was shown chromatographically) the

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following products were isolated and identified in the form of methyl esters: IIIb, IVb, Vb, VIb, VIIb, VIIIb and IXb (see the Scheme).

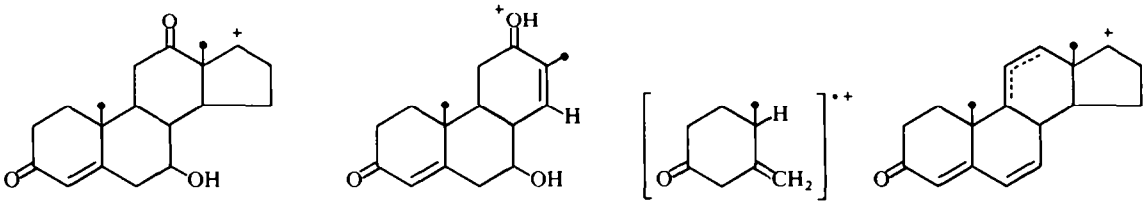
Methyl 7α-hydroxy-3,12-diketo-Δ⁴-cholenate (IIIb)

The IR and UV spectra reveal the presence of a Δ⁴-3-keto-group, an OH group, unconjugated ketone and an ester group. The mass spectrum (Fig. 1a) displays the molecular ion peak (*m/e* 416), the abundant peak M-H_2O (*m/e* 398), as well as peaks with *m/e* 301 (M-115, ion *a*) and 283 (ion *a*—H_2O). On this basis, the molecule of ester IIIb contains an OH group and the side chain of cholic acid.

Peaks with *m/e* 261 (ion *b*) and 243 (ion *b*—H_2O) are due to elimination of ring D with the migration of a H-atom to the charged fragment as is characteristic of 12-ketosteroids.[13]

Finally, the presence of a peak with *m/e* 124 (ion *c*) gives further confirmation of the presence of the Δ⁴-3-keto-grouping[14] in the molecule.

The structure of methyl 7α-hydroxy-3,12-diketo-Δ⁴-cholenate was therefore ascribed to compound IIIb.



a *b* *c* *d*

Methyl 7α-hydroxy-3,12-diketo-Δ⁴-bisnorcholenate (IVb)

The UV data show the presence of the Δ⁴-3-keto-grouping. The IR spectrum reveals the presence of hydroxy and ester groups, a keto group in a 6-membered ring and the Δ⁴-3-keto-grouping. Comparison of the mass spectra of the esters IIIb and IVb (Fig. 1b) shows that the peaks of the molecular ion (*m/e* 388) as well as of the fragments containing the side chain (e.g. M-H_2O, *m/e* 370) are shifted by 28 mass units to the low mass region, whereas *m/e* values of the fragments deprived of the side chain remain unchanged (ion *a*, *m/e* 301; ion *a*—H_2O, *m/e* 283; ion *b*, *m/e* 261; ion *b* —H_2O, *m/e* 243; ion *c*, *m/e* 124). This suggests that the esters IIIb and IVb differ only in the length of the side chain which in IVb contains two methylene units less. Based on the data, compound IVb was ascribed the structure of methyl 7α-hydroxy-3,12-diketo-Δ⁴-bisnorcholenate. As mentioned, there is no evidence of side chain degradation in cholic acid by mycobacteria. Thus, the possibility of this degradation in the molecule of a bile acid (on the example of cholic acid) by a species of Mycobacterium has been demonstrated.

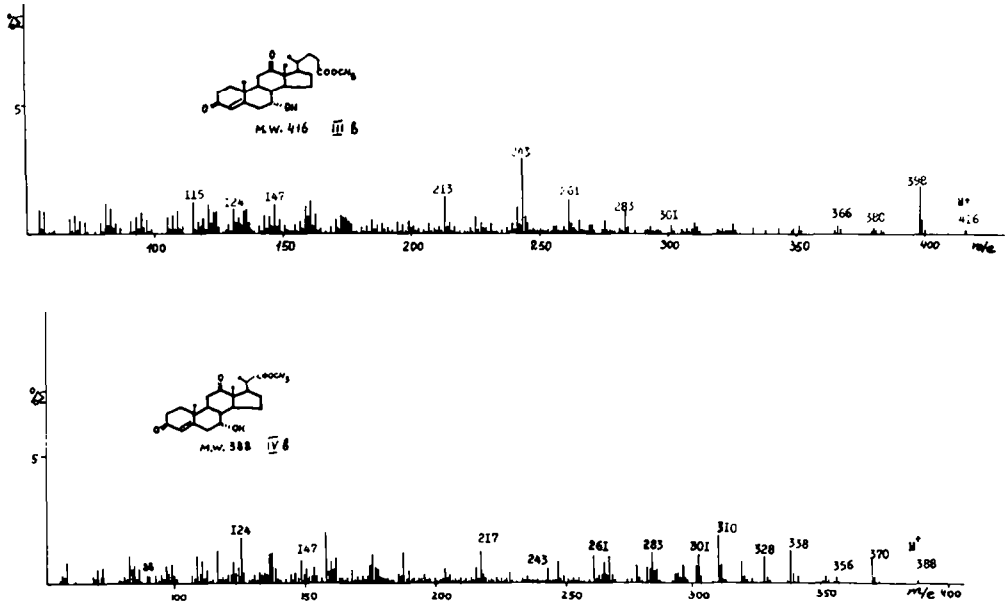


FIG. 1. Mass spectra of:
(a) methyl 7α-hydroxy-3,12-diketo-Δ^4-cholenate (IIIb)
(b) methyl 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenate (IVb)

Methyl-7α-hydroxy-3,12-*diketo*-$\Delta^{4,8(9)}$-*bisnorcholadienate* (Vb)

The spectral features of the compound Vb are similar to those of IVb. The IR and UV spectra reveal the presence of the Δ^4-3-keto-grouping, unconjugated ketone and hydroxy and ester groups. In the mass spectrum (Fig. 2a) of the ester Vb the *m/e* values of the molecular ion as well as of the fragments containing rings B and C are shifted by 2 mass units to the low mass region when compared with the spectrum of IVb, whereas *m/e* of the fragment *c* (*m/e* 124) remains unchanged. Indeed, the peak M-H_2O has *m/e* 368, the peaks due to elimination of the side chain or the ring D (+H-atom) from M^+ or M-18 have *m/e* 299 (M- the side chain), 281 (*m/e* 299—H_2O), 259 (M- the ring D + H-atom) and 241 (*m/e* 259—H_2O), respectively. These facts indicate that the esters IVb and Vb have similar structures but differ in the presence of an additional double bond either in the ring B or C in the molecule of Vb.

To determine the double bond position the NMR spectra of both esters (IVb and Vb) were taken in pyridine and DMSO. The NMR spectra of both compounds in DMSO display the signal of a single vinyl proton at 5·70 ppm for IVb and at 5·68 ppm for Vb. This suggests that the Vb has a tetrasubstituted double bond which therefore can only be in the positions 8,9; 8,14 or 7,8. The chemical shifts of the angular Me groups (in pyridine) are 1·01 ppm (18-Me) and 1·31 (19-Me) for Vb and 1·05 ppm and 1·20 ppm for IVb, respectively. A paramagnetic shift of 0·11 ppm is thus observed for the 19 Me group in passing from IVb to Vb. This suggests the $\Delta^{8(9)}$-position for the double bond since a $\Delta^{8(14)}$-double bond should cause a diamagnetic shift for the 19-Me group.[15]

Furthermore, the NMR spectra of both Vb and IVb in DMSO display the signal of the proton at the C-atom bonded to the OH group (4·15 ppm for Vb and 4·30 ppm for IVb).

On this basis, the compound Vb has the structure methyl 7α-hydroxy-3,12-diketo-$\Delta^{4,8(9)}$-bisnorcholadienate, but the low intensity of M-H_2O peak when compared with the analogous peak in the spectrum of IVb is in contradiction with the structure suggested since in this case (the allylic alcohol system) easy dehydration should proceed under electron impact as well as by the action of chemical reagents. In fact dehydration of Vb with phosphorous oxychloride in pyridine (100°) yielded no dehydration product with a $\Delta^{4,6,8}$-trienic system, but resulted in an isomeric ester with the $\Delta^{4,6}$-diene system. The mass spectrum of the latter was identical with that of VIb (see Fig. 2b).*

Methyl 7-hydroxy-3,12-*diketo*-$\Delta^{4,6}$-*bisnorcholadienate* (VIb)

UV data reveal the presence of the $\Delta^{4,6}$-3-keto-grouping (λ_{max} 285 mμ). The presence of hydroxy and ester groups, a keto-group in a 6-membered ring and $\Delta^{4,6}$-3-keto-grouping are confirmed by the IR spectrum.

In the mass spectrum of VIb (Fig. 2b) the peak of the molecular ion (*m/e* 386) is

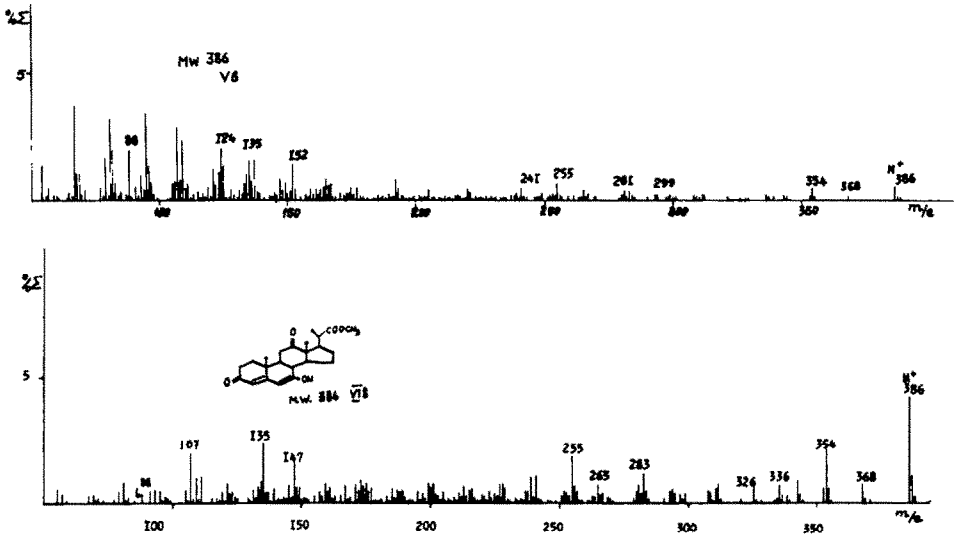


FIG. 2. Mass spectra of:
(a) methyl ester Vb
(b) methyl 7-hydroxy-3,12-diketo-$\Delta^{4,6}$-bisnorcholadienate (VIb)

the most abundant whereas (in contrast to the esters IIIb and IVb) the peak M-H_2O (*m/e* 368) is of low intensity. This suggests that the OH group in VIb is located at the double bond. The presence of the $\Delta^{6,7}$ double bond is also confirmed by the extremely low intensity of the peak with *m/e* 124 (*c*) in the mass spectrum of VIb. The latter

* As far as we know, microbiological dehydration of steroids in position 8,9 has not been published.

spectrum displays also peaks with *m/e* 299 and 88 ([EtCOOMe])$^{+\cdot}$[16] which characterize the length of the side chain.

Methyl 12α-*hydroxy*-3-*keto*-$\Delta^{4,6}$-*choladienate* (VIIb) *and* 12α-*hydroxy*-3-*keto*-$\Delta^{4,6}$-*bisnorcholadienate* (VIIIb)

The UV spectra of both esters display the absorption max at 280 mμ which is characteristic of the $\Delta^{4,6}$-3-keto-grouping. The IR spectrum of VIIIb reveals the presence of hydroxy and ester groups and the $\Delta^{4,6}$-3-keto-grouping. The cleavage of the molecular ions of the esters VIIb and VIIIb (*m/e* 400 and 372, respectively) under electron impact is accompanied in both cases by successive elimination of H_2O ($M-H_2O$, *m/e* 382 and 354, respectively) and the side chain. The latter process results in the formation of the fragment (*d*) with *m/e* 267, the corresponding peak in the spectra of both compounds VIIb and VIIIb (Fig. 3a, b) being the most abundant.

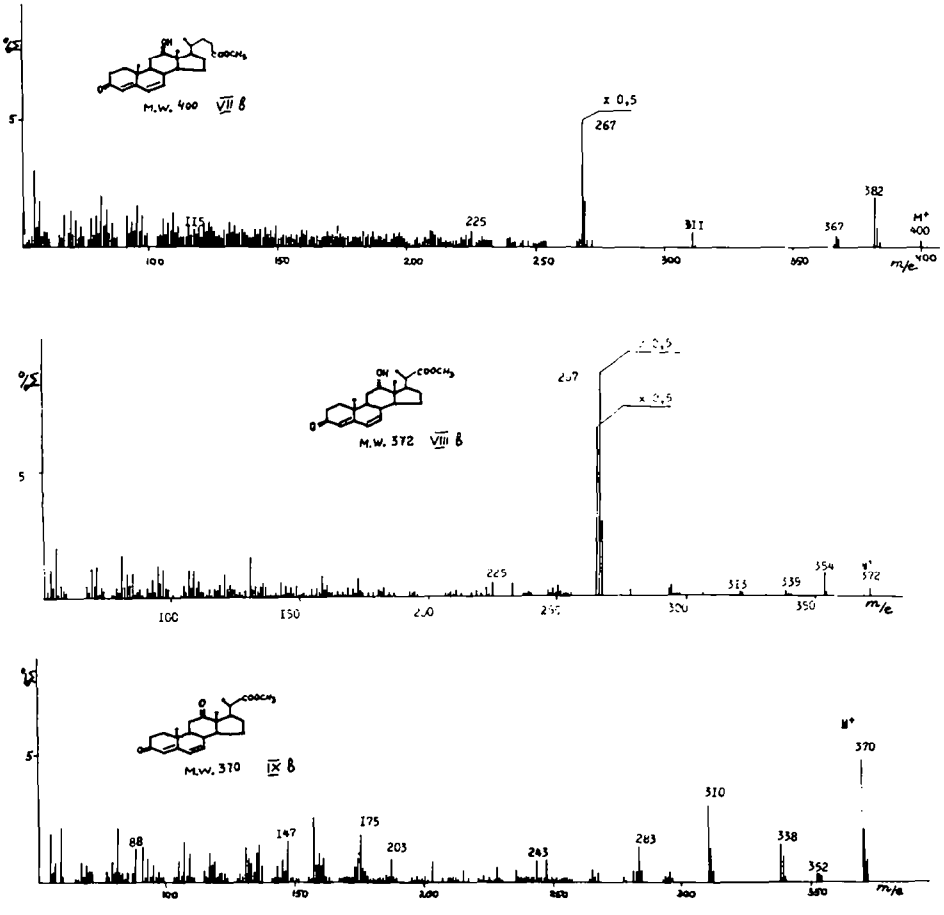
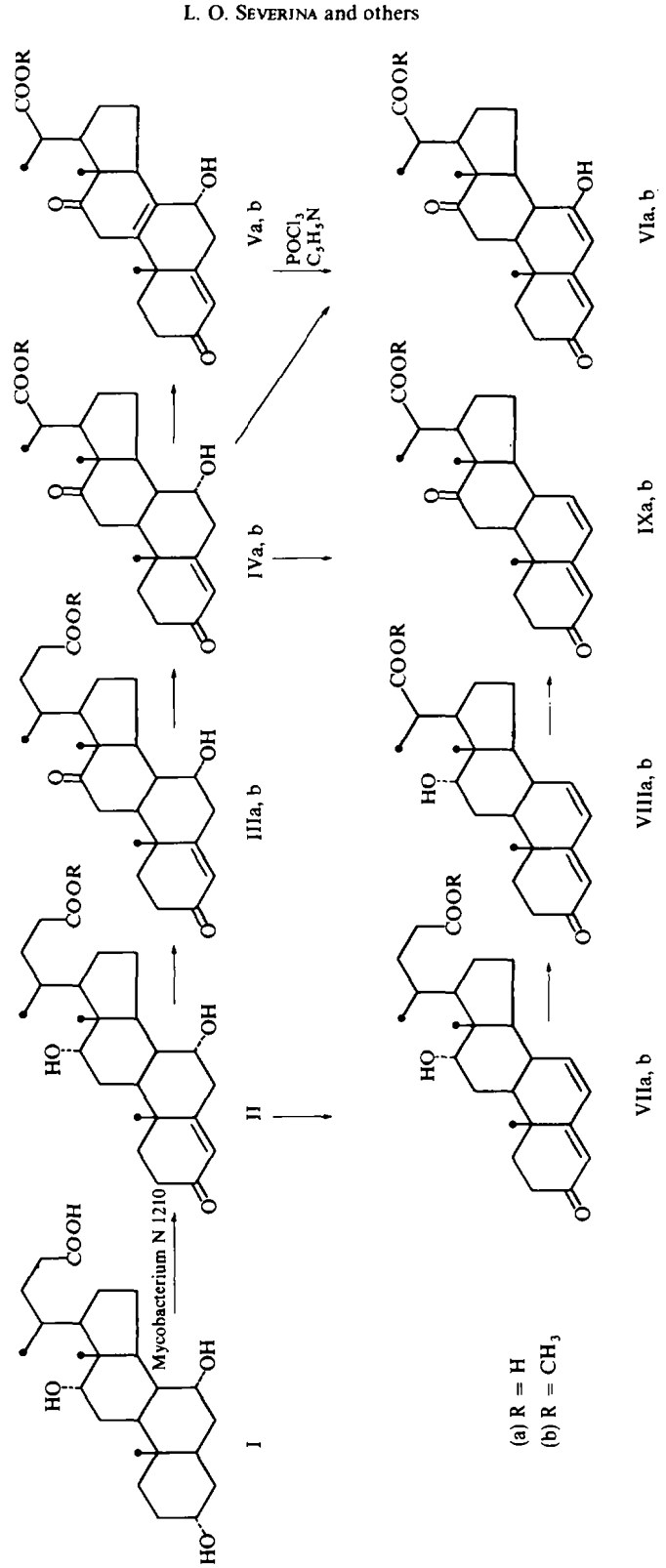


FIG. 3. Mass spectra of:
(a) methyl-12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienate (VIIb)
(b) methyl 12α-hydroxy-3-keto-$\Delta^{4,6}$-bisnorcholadienate (VIIIb)
(c) methyl 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienate (IXb)

Scheme of cholic acid transformation by the culture mycobacterium N 1210



This leads to the conclusion that the esters VIIb and VIIIb have similar structures and differ only in the length of the side chain (in the ester VIIIb the side chain is shorter by two methylene units).

Methyl 3,12-*diketo*-$\Delta^{4,6}$-*bisnorcholadienate* (IXb)

IR and UV data reveal the presence of a $\Delta^{4,6}$-3-keto-grouping, a ketogroup in a 6-membered ring and an ester group and the absence of hydroxy groups. The mass spectrum of IXb (Fig. 3c) differs from the spectra of hydroxyesters IIIb–VIIIb by the high intensity of its molecular ion peak (*m/e* 370) which in this case is the most abundant one in the spectrum. At the same time the peak M-H_2O (*m/e* 352) is of low intensity (about 8% of the intensity of M^+). This is in agreement with the absence of an OH group in the molecule of IXb. The spectrum of IXb displays also the peak M-87 (*m/e* 283) which indicates the length of the side chain, and a peak with *m/e* 243 (M- the ring D + H-atom) due to the cleavage characteristic of 12-keto-groups.[13]

These data suggest the structure of methyl 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienate for IXb.

Based on these results we put forward the scheme of cholic acid transformation by the culture Mycobacterium (see opposite page).

Methyl 7α-12α-dihydroxy-3-keto-Δ^4-cholenate (IIb) has not been isolated, but we suppose its formation to take place as evidenced by the isolation of 12α-hydroxyacid methyl esters.

Work on isolation and identification of other minor products is now in progress.

EXPERIMENTAL

The m.ps were determined on a Kofler block. The UV spectra were taken in alcohol solns on a spectrophotometer SF-4; IR spectra were taken in vaseline oil on spectrophotometers UR-10 and Hilger H-800; the NMR spectra were taken on the spectrometer INM-C-60.

The mass spectra were taken on Soviet commercial instruments MX-1303 and MX-1306 with the glass inlet system for direct insertion of the sample into the ion source and with temp stabilization (±1°). The ionizing energy was 24–30 eV and the temp was 110° (IIIb, IVb, VIb), 200° (Vb) (MX-1306) and 150–170° (VIIb, VIIIb, IXb) (MX-1303).

Chromatography was carried out on alumina (III and IV activity). For separation of methyl esters on paper (chromatographic paper C) the following systems were used:

1. hexane:benzene:methanol:water 6:6:2:1
2. hexane:benzene:methanol:water 20:2:2:1

Fermentation. 4 l. of nutrient medium [$(NH_4)_2SO_4$—2·0 g; K_2HPO_4—1·0 g; $MgSO_4 \cdot 7H_2O$—0·5 g; K_2CO_3—1·0 g; $FeCl_3 \cdot 6H_2O$—0·01 g; cholic acid—1·0 g; distilled water—1000 ml; after sterilization pH 7] were inoculated by 3-day culture Mycobacterium N 1210 (previously grown on agarized medium) in a fermenter provided with barboter (1 l. of air pro 1 l. of medium in 1 min) and stirrer (180 rpm) at 28°. The course of transformation was controlled by paper chromatography in the system benzene:ethanol:water:acetic acid (2:1:2:0·2). In 70 hr of fermentation the chromatographic control manifested the absence of cholic acid in the cultural medium and the presence of a mixture of compounds absorbing UV light. The cultural medium was adjusted to pH 7·5 with dil $NaHCO_3$ aq and centrifuged at 3000 rpm for 1 hr. The supernatant liquor was evaporated *in vacuo* to 400 ml at a temp not higher than 50°, adjusted to pH 2 with 2N HCl and extracted 4 times with double its volume of ether. Extracts were combined, dried with $MgSO_4$, evaporated *in vacuo* to about 70 ml and the ppt formed was filtered off (ppt A, 780 mg). Crystallization from EtOAc–EtOH yielded 520 mg of substance B, m.p. 238–244°. A second crystallization gave 280 mg of C, m.p. 254–258° which was methylated in a usual manner with an etheral soln of diazomethane. The resulting mixture of methyl esters (substance EC) was preparatively separated on a thin layer of alumina.

Methyl 7α-*hydroxy*-3,12-*diketo*-$\Delta^{4,8(9)}$-*bisnorcholadienate* (Vb). Successive separation of the substance EC on a thin layer of alumina in the systems EtOAc:ether (4:3, 5:2) and pure EtOAc resulted in isolation of 92 mg of a compound, m.p. 276–282° (R_f 0·24 in the system 1). It was crystallized twice from EtOAc–EtOH and once from EtOH yielding 29 mg of Vb, m.p. 290–293°, λ_{max} 241 mμ (log ε 4·20); IR spectrum: 3400, 1733, 1704, 1670 and 1618 cm^{-1}.

Methyl 7α-*hydroxy*-3,12-*diketo*-Δ^{4}-*bisnorcholenate* (IVb). The ester IVb was isolated by successive TLC of the substance EC on alumina in the systems EtOAc:ether (4:3 and 5:2). 61 mg of a compound, m.p. 249–252° (R_f 0·49 in the system 1), was obtained. It was crystallized twice from EtOAc–EtOH yielding 24 mg of IVb, m.p. 258–260°, lit. values:[4,5] m.p. 245–246°, λ_{max} 242 mμ (log ε 4·22); IR-spectrum: 3505, 1738, 1700, 1670 and 1620 cm^{-1}.

In addition, the following compounds were isolated: 12 mg of IIIb, m.p. 182–186° (R_f 0·65 in the system 1), after two recrystallizations from EtOAc, m.p. 188–191°; 3 mg of E, m.p. 205–210° (R_f 0·18 in the system 1), after crystallization from EtOAc, m.p. 215–218° and 5 mg of F, m.p. 188–192° (R_f 0·33 in the system 1).

The mother liquor from the isolation of C was evaporated to dryness *in vacuo* and methylated with diazomethane yielding 243 mg of a mixture of methyl esters. 100 mg of the mixture were separated on a thin layer of alumina in the systems EtOAc:ether (4:3, 5:2) and EtOAc. The following compounds were isolated: 8 mg of Vb, m.p. 275–280° (R_f 0·22 in the system 1), after two recrystallizations, m.p. 289–292°, no m.p. depression with the previous sample of Vb, their IR spectra were identical; 27 mg of IVb, m.p. 247–253°, after crystallizations, m.p. 258–260°, no m.p. depression with authentic sample, their IR spectra were identical.

Methyl 7α-*hydroxy*-3,12-*diketo*-Δ^{4}-*cholenate* (IIIb). In the system EtOAc:ether (3:4 and 4:3) the compound (25 mg) with m.p. 181–185° (R_f 0·66 in the system 1) was isolated which proved to be identical with IIIb (identical IR spectra, no m.p. depression). Double crystallization from EtOAc yielded 12 mg, IIIb, m.p. 188–190°, lit. values:[7] m.p. 191–193°, λ_{max} 242 mμ (log ε 4·16); IR spectrum: 3540, 1739, 1715, 1665 and 1618 cm^{-1}. (Found: C, 72·40; H, 8·85. Calc. for $C_{25}H_{36}O_5$: C, 72·08; H, 8·71%.)

Methyl 7-*hydroxy*-3,12-*diketo*-$\Delta^{4,6}$-*bisnorcholadienate* (VIb). Separation in the system EtOAc:ether (3:4 and 4:3) resulted in isolation of 7 mg of VIb, m.p. 231–238° (R_f 0·75 in the system 1), after two crystallizations from EtOAc, m.p. 238–240°; λ_{max} 285 mμ; IR spectrum: 3530, 1738, 1715, 1665, 1623 and 1590 cm^{-1}.

In addition, the following compounds were isolated by separation of the mixture on a thin layer of alumina in the systems EtOAc:ether (3:4, 4:3 and 5:2): 10 mg of the ester with m.p. 188–192°, after two crystallizations from EtOAc, m.p. 193–195°, no m.p. depression with a sample of F, the same chromatographic mobility; 2 mg of the ester E, m.p. 204–215°.

The mother liquor after isolation of B was evaporated to dryness *in vacuo* and crystallized from EtOAc:ether. The crystals (D, 46 mg) were methylated with diazomethane yielding 49 mg of a mixture of methyl esters. The latter was separated on a thin layer of alumina in the systems EtOAc:ether (3:4, 4:3). The following compounds were isolated: 4 mg of IVb, after two crystallizations from EtOAc–alcohol, m.p. 256–258°, no m.p. depression with the authentic sample, identical IR spectra; 15 mg of IIIb, m.p. 182–188°, after two crystallizations from EtOAc, m.p. 189–191°, no m.p. depression with authentic sample, identical IR spectra; 6 mg of F, m.p. 190–192°, identical in chromatographic mobility (in the system 1) with the above described sample; and 3 mg of VIb, m.p. 231–238°, identical in chromatographic mobility with the above described sample.

The mother liquor after separation of the ppt A was evaporated to dryness *in vacuo* and the yellow-brown oil obtained (193 mg) was methylated by diazomethane. The resulting mixture of methyl esters (EA) was separated on a thin layer of alumina.

Methyl 12α-*hydroxy*-3-*keto*-$\Delta^{4,6}$-*bisnorcholadienate* (VIIIb). Successive separation of the mixture EA on the systems EtOAcether (3:4, 2:5) and pure ether yielded 7 mg of VIIIb, as an oil which became crystalline after scratching with cold ether and then was twice crystallized from ether. The pure sample of VIIIb (R_f 0·72 in the system 2) has m.p. 156–158°, λ_{max} 280 mμ; IR spectrum: 3480, 1740, 1658, 1625 and 1590 cm^{-1}.

Methyl 12α-*hydroxy*-3-*keto*-$\Delta^{4,6}$-*choladienate* (VIIb). Similarly from the mixture EA an oil (5 mg) was isolated, which after two crystallizations from ether–EtOAc yielded pure VIIb (R_f 0·76 in the system 2), m.p. 180–182°, lit. values:[9,10] m.p. 182–184°, λ_{max} 280 mμ.

Methyl 3,12-*diketo*-$\Delta^{4,6}$-*bisnorcholadienate* (IXb). A semicrystalline compound (22 mg) was isolated from the mixture EA. Recrystallization from EtOAc:ether and from EtOAc yielded pure IXb, m.p. 180–

182°, lit. values:[5] m.p. 181–183°, λ_{max} 280 mμ (log ε 4·46), (R_f 0·89 in the system 2); IR spectrum: 1735, 1715, 1655, 1620 and 1582 cm^{-1}.

In addition, separation of the mixture EA gave 4 mg of a compound identical in chromatographic mobility with IIIb and 2 mg of a compound with R_f close to that of VIb.

Attempted dehydration of the ester Vb. A mixture of Vb (2 mg), dry pyridine (2 ml) and distilled $POCl_3$ (4 mg) was heated at 100° for 7 hr. After this period, chromatographic control revealed only a small amount of the initial compound and a significant quantity of a less polar product. The reaction mixture was poured into 8 ml dil HCl (1:4), kept for 1 hr at room temp and then thoroughly extracted with EtOAc. The combined extracts were washed with a Na_2CO_3 aq and dried over Na_2SO_4. The yellow oily residue obtained after evaporation of the solvent *in vacuo* was separated on a thin layer of alumina in the system EtOAc:ether 1:1. The less polar (when compared with starting material) product was eluted with the mixture EtOAc–alcohol and crystallized from EtOAc yielding the compound (m.p. 240–243°, λ_{max} 285 mμ) which was identical with VIb in chromatographic mobility and showed the same mass spectrum.

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