Tetrahedron Vol 25, pp. 5617 to 5622 Pergamon Press 1969 Printed in Great Britain

THE TRANSFORMATION OF DESOXYCHOLIC ACID BY THE CULTURE *MYCOBACTERIUM MUCOSUM* 1210

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(*Received in the UK* 27 *May* 1969; *Accepted for publication* 1 *August* 1969)

Abstract—The enzymatic transformation of desoxycholic acid by *Mycobacterium mucosum* 1210 has been studied. 3,12-diketo-Δ^4-bisnorcholenic and 9α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid have been isolated in the form of methyl esters.

CERTAIN cultures of Actinomycets, Mycobacteria and Fungi are capable of transforming cholic acid into different products[1–6] when this acid is the only source of carbon. The transformation includes the oxidation of OH groups to CO groups, the Δ^4-dehydrogenation of the ring A and sometimes the β-oxidation of the side chain.[3–6]

In 1942 Hughes and Schmidt obtained 3,12-diketocholanic acid by the fermentation of desoxycholic acid with intestinal bacterium *Alcaligenes faecalis*. Leifson, *et al.* noticed a toxic action of desoxycholic acid on some bacteria and actinomycetes,[8–10] and we have shown that *Mycobacterium mucosum* 1210 transforms choloric acid when it is the only source of carbon.[11,12] Compounds with a shortened side chain and with the Δ^4-3-keto-grouping in the ring A were found among the conversion products.[13–14] The present paper deals with the enzymatic transformation of desoxycholic acid by the culture *Mycobacterium mucosum* 1210.

RESULTS AND DISCUSSION

After completing the transformation process two compounds with m.p. 200–202° and 225–228° were isolated as the main components (in the form of methyl esters) from the ethereal extract of the culture liquid. The first compound was shown to be methyl 3,12-diketo-Δ^4-bisnorcholenate (I) and the second methyl 9α-hydroxy-3,12-diketo-Δ^4-bisnorcholenate (II).

Compound I. The IR spectrum of the ester I revealed the presence of the Δ^4-3-keto-grouping in a 6-membered ring with an ester bond and no OH group. The mass spectrum of I displays the molecular peak (*m/e* 372), the abundant *m/e* 124 peak (ion **a**) characteristic of Δ^4-3-keto-steroids,[15] the peaks M-87 (ion **b**, *m/e* 285), M-88 (*m/e* 284) and *m/e* 88 $(EtCOOCH_3)^{+\cdot}$ due to the loss of the side chain, as well as the peak of the tricyclic fragment **c** (M^+ − ring D + H-atom) with *m/e* 245 (the migration of an H-atom to the charge fragment is due to the presence of 12-keto-function.[16]

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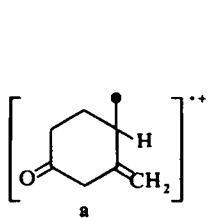
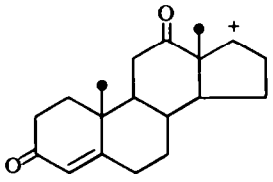
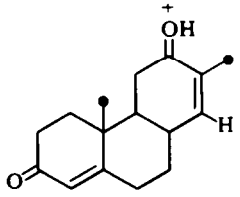
The comparison of the mass spectra of I and methyl 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienate (Ia) studied earlier[13] shows that the *m/e* values of M^+ and the main characteristics fragments containing the ring B (ions **b**, **c**, etc) in the mass spectrum of I are shifted by 2 mu to the high mass region. This fact as well as the presence of the *m/e* 124 peak in the spectrum of the ester I suggests that I is the analog of the ester Ia and hence has the structure of methyl 3,12-diketo-Δ^4-bisnorcholenate.



a



b



c

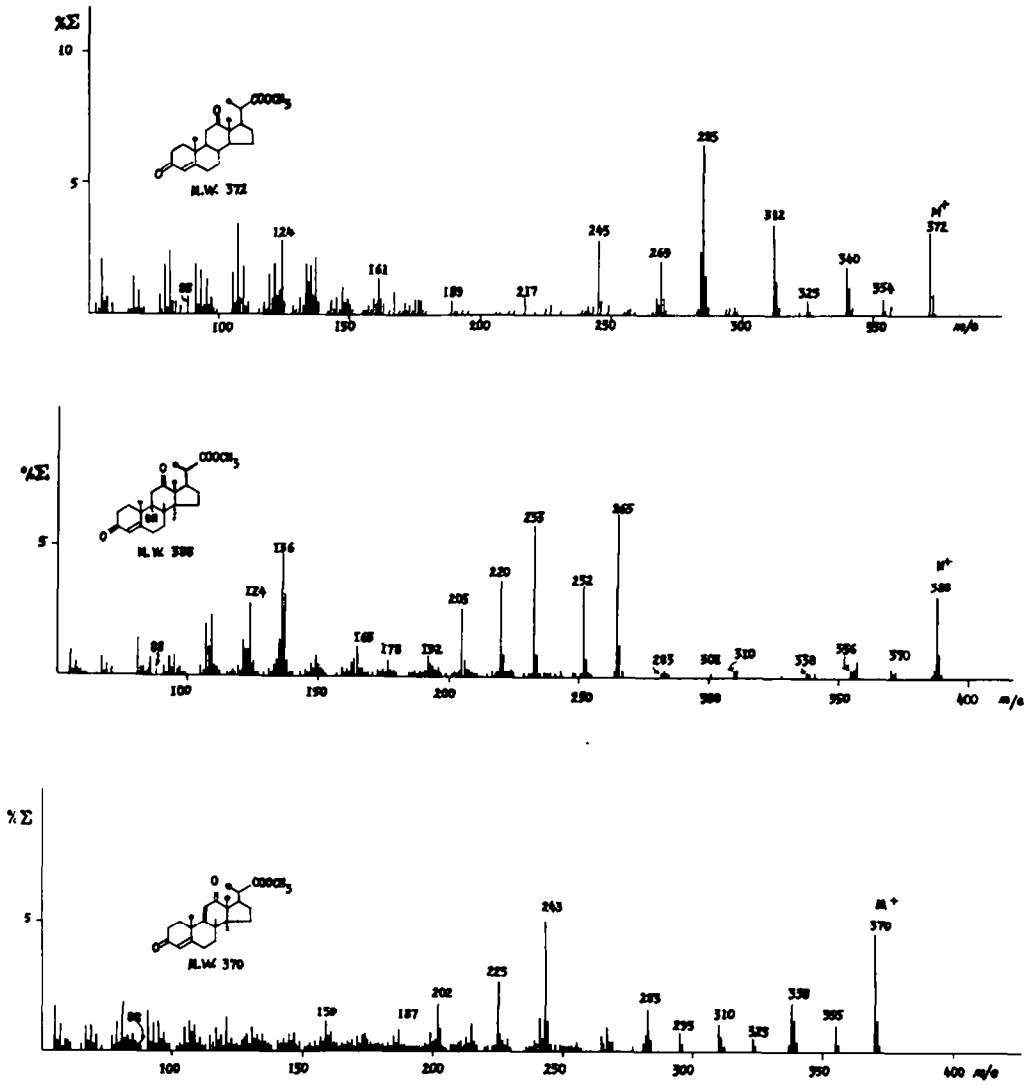
As in the case of Ia, the abundant peak due to the expulsion of the $HCOOCH_3$ molecule (M-60, *m/e* 312) is observed in the spectrum of I. The further degradation of the ion M-$HCOOCH_3$ leads (as confirmed by the corresponding metastable peak, Table 1) to the *m/e* 269 fragment which is apparently due to the expulsion of the CO and the CH_3-radical[17] from the ring C. In contrast to Ia, abundance of the *m/e* 269 peak in the spectrum of I is very significant (about 70% of that of M^+).

TABLE 1.

Compound	Transition	*m/e* of the metastable peak	
		Found	Calc
I	372 → 285	218	218·1
	312 → 269	232	231·8
	357 → 325	296	295·8
II	388 → 370	353	352·8
	388 → 356	327	326·6
	370 → 338	309	308·7
	388 → 265	181	181
	388 → 252	163·5	163·5
	252 → 220	192	192

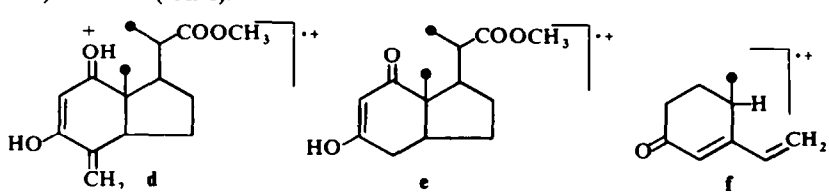
Compound II. The IR spectrum of the ester II revealed the presence of the Δ^4-3-keto-grouping in the 6-membered ring, an ester bond and an OH group. The latter could not be acetylated by the acetic anhydride in pyridine and was stable to oxidation by CrO_3 in AcOH. This suggested its tertiary character.

The mass spectrum of II displays the molecular peak (*m/e* 388), the small peak M-H_2O (*m/e* 370), the peaks which characterize the length of the side chain (M-87, *m/e* 301; M-87-H_2O, *m/e* 283; $EtCOOCH_3^+$, *m/e* 88) and the peaks which confirm the presence of the Δ^4-3-keto-grouping (ion **a**, *m/e* 124), the 12-keto-function (M-ring D + H-atom-H_2O, *m/e* 243) and $COOCH_3$ group (M-CH_3OH, *m/e* 356; M-H_2O—$HCOOCH_3$, *m/e* 310).



Consequently compound II is methyl hydroxy-3,12-diketo-Δ^4-bisnorcholenate. The position of the OH group was established as follows:

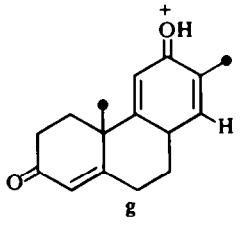
In contrast to the spectrum of methyl 7α-hydroxy-3,12-d iketo-Δ^4-bisnorcholenate,[13] the mass spectrum of the hydroxyester II displays the abundant peaks at *m/e* 265 (ion **d**) and 252 (ion **e**).



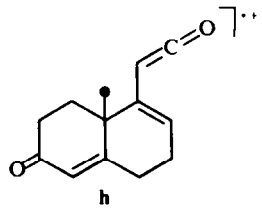
The presence of the corresponding metastable peaks (Table 1) reveals that the ions **d** and **e** are formed directly from the ion M^+. These fragments contain the rings C and D since their further degradation, similar to that of the molecular ion, is accompanied by the loss of the MeOH or AcOH molecules or the whole side chain and results in the ions 233 and 220, 205 and 192, 178 and 165, respectively. In other words, the formation of the ions **d** and **e** deals with the cleavage of the ring B: 9, 10 and 6, 7 (ion **d**) or 9,10 and 7,8 (ion **e**). In addition, the breakdown of the bonds 9,10 and 7,8 with the localization of the positive charge in the fragment of the ring A leads to the ion **f** (*m/e* 136) which is also one of the most abundant in the spectrum.

Since the OH group usually initiates the processes of α-cleavage,[18] the OH group is in the 9- or 8- position of the molecule of II. Recently,[19] the mass spectrum of 9α-hydroxyprogesterone was published. The fragmentation of the latter under electron impact is similar to that described for the ester II.

Dehydration of the ester II by *p*-toluenesulfonic acid in benzene followed by TLC on Al_2O_3 was carried out for final proof of the position of the OH group. The IR spectrum of compound III isolated reveals the absorption bands of the ester bond and the α,β-unsaturated ketone while the bonds of the OH group and the saturated ketone are absent. The analysis of the mass spectrum of III shows that this compound has the structure of methyl 3,12-diketo-$\Delta^{4,9(11)}$-bisnorcholadienate. Indeed, the spectrum of the ester III displays the molecular peak (*m/e* 370), the peaks due to the loss of the side chain (*m/e* 283 and 88), as well as the peak of the tricyclic fragment (M-ring D + H-atom, ion **g**, *m/e* 243) which is the most abundant one in the spectrum. The extensive degradation of M^+ with the formation of the fragment **g** is characteristic of $\Delta^{9(11)}$-12-keto steroids.[20] The presence of the *m/e* 202 peak (ion **h**) in the spectrum of the ester III is also in accordance with the $\Delta^{9(11)}$-12-keto grouping.



g



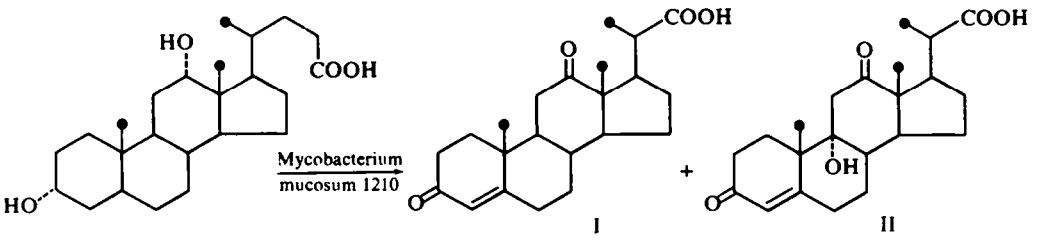
h

Heating of the ester II with alkali with subsequent esterification gave ester III. The formation of the latter is evidence that the compound II is a β-ketol and the OH group is therefore located in the position 9.

It has been shown,[21–24] that the OH group incorporated microbiologically is at the same position previously occupied by the hydrogen atom and, therefore, compound II is methyl 9α-hydroxy-3,12-diketo-Δ^4-bisnorcholenate.

Microbiological hydroxylation of a steroid molecule occurs often and is usually followed by the splitting of the ring B and the formation of 9,10-seco compounds.[25–27]

The transformation of desoxychlolic acid by the culture *Mycobacterium mucosum* can therefore be represented by the following scheme.



EXPERIMENTAL

The m.ps were determined on a Koffler block. The IR spectra were run on the spectrophotometer UR-10 in the paste with vaseline oil.

The mass spectra were taken on the Soviet commercial instrument MX-1303 furnished with the all-glass system allowing direct sample inlet into the ion source (± 1°, temp stabilization) at the ionizing voltage 24–30 V and the temperatures 170° (I), 200° (II), 155° (III).

The chromatography was carried out on alumina (III degree of activity according to Brockman).

The fermentation was carried out in Erlenmeyer flasks under shaking (160 Emp) at 27°. The medium consisted of $(NH_4)_2SO_4$—2·0 g, K_2HPO_4—1·0 g, $MgSO_4$. 7 H_2O—0·5 g, K_2CO_3—1·0 g, $FeCl_3$. 6 H_2O—0·01 g, desoxycholic acid—1·0 g, distilled water—1000 ml, pH 7 (after sterilization). 20 flasks each containing 50 ml of the medium were inoculated by the 3-day culture of *Mycobacterium mucosum* 1210 previously grown with cholic acid on the agar medium. After fermentation for 8 days the shaking was stopped, the content of the flasks was combined, adjusted to pH 2 with 2N HCl and extracted four times with ether. The extract was dried with $MgSO_4$ and evaporated to dryness *in vacuo*. The yellow-brown oil obtained (705 mg) was methylated by diazomethane and the mixture of methyl esters was preparatively separated on a thin layer of alumina.

Methyl 3,12-*diketo*-Δ^4-*bisnorcholenate* (I) *and* 9α-*hydroxy*-3,12-*diketo*-Δ^4-*bisnorcholenate* (II)

The separation of the mixture of methyl ester on a thin layer of alumina in ether and then in the systems ether–EtOAc (3:1 and 3:2) resulted in isolation of 112 mg of the less polar compound. Double crystallization from EtOAc gave 46 mg of the ester I with m.p. 200–202°. IR spectrum: 1749, 1710, 1668 and 1612 cm^{-1}. 70 mg of the compound were collected from the more polar fraction. Double crystallization from EtOAc gave 20 mg of II with m.p. 225–228°. IR spectrum: 3480, 1740, 1710, 1680 and 1617 cm^{-1}.

Dehydration. The ester II (8 mg) was dehydrated by boiling for 20 min with 2·4 mg *p*-toluenesulfonic acid in 0·5 ml benzene. The mixture was separated by TLC on alumina. The crystallization of the less polar compound (4·1 mg) from the mixture EtOAc–ether gave III with m.p. 186–188° IR spectrum: 1740, 1673, 1620 cm^{-1}.

The ester was dehydrated by boiling for 10 min with KOH in pyridine water soln. After the usual treatment, esterification (diazomethane) and TLC, the ester III was isolated, which was identical in every respect with the compound described above.

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