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MICROBIAL HYDROXYLATION OF THE CHOLESTEROL SIDE CHAIN

I. I. ZARETSKAYA,* L. M. KOGAN,* O. B. TIKHOMIROVA,* JH. D. SIS,† N. S. WULFSON,‡ V. I. ZARETSKII,‡ V. G. ZAIKIN,‡ G. K. SKRYABIN† and I. V. TORGOV*

Institute for Chemistry of Natural Products and Institute of Microbiology of the USSR Academy of Sciences

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Abstract—Fermentation of cholesterol with the culture of *Mycobacterium sp.* 2104 yielded 27-hydroxy-cholest-4-en-3-one which is the first case of microbiological hydroxylation of a sterol side chain.

IT WAS shown earlier[1] that the oxidation of cholesterol (I) to cholest-4-ene-3-one (II) took place during fermentation reactions with different microorganisms (Actinomycetes, Proactinomycetes, Mycobacteria). In these reactions we noticed the formation of a substance which was more polar than cholesterol and whose structure was not established.

In this paper we report the results obtained during the course of the detailed study of transformation of cholesterol by a culture of *Mycobacterium sp.* 2104 which was isolated from soil on the agar medium with cholic acid as the only source of carbon.

The fermentation of this culture for 48 hours with cholesterol (I) gave cholest-4-en-3-one (II) in 32% yield (based on cholesterol used) and a substance III which is more polar in character than cholesterol, with the m.p. 138–139·5° (1–1·5% yield).

This substance is easily detected by spraying the chromatograms with Lugol reagent when it gives an intense blue coloration, unlike cholesterol. The IR and UV spectra of this compound show the presence of a Δ^4-3-keto group and an OH group. The ketol III on acetylation at room temperature yields the monoacetate IV.

The mass spectrum of the compound III (Fig. 1a) reveals the intense molecular peak at *m/e* 400 as well as peaks at *m/e* 124 (ion *a*) and 358 $(M\text{-}CH_2CO)^+$ (ion *b*) which are characteristic of Δ^4-3-ketosteroids.[2] A comparison of the spectra of ketol III and cholest-4-en-3-one (II) indicates that the former possesses an OH group in the side chain. This is confirmed by the fact that the mass numbers of the molecular ion peak as well as of the peaks of all ions containing the side chain (at *m/e* 385 $(M\text{-}CH_3)^+$, 358, 277 (ion *c*)) are increased by 16 units as compared with those in the spectrum of cholestenone (II), while the peaks at *m/e* 299 (ion *d*), *m/e* 271 (ion *e*), *m/e* 229 (ion *f*) and *m/e* 124 (*a*) remain unaffected.

There is a noticeable peak at *m/e* 73 corresponding to the $(CH_2OCOCH_3)^+$ ion in the low mass-number range of the mass spectrum of the monoacetate IV (Fig. 1b). This proves that the ketol III is a primary alcohol. In addition, the fragmentation pattern of 24-hydroxy-cholest-4-en-3-one induced by electron impact differs very much from that of ketol III. This is yet another proof of the primary character of the

* Laboratory of Steroid Chemistry of Institute for Chemistry of Natural Products.
† Institute of Microbiology.
‡ Laboratory of Mass Spectrometry of Institute for Chemistry of Natural Products.

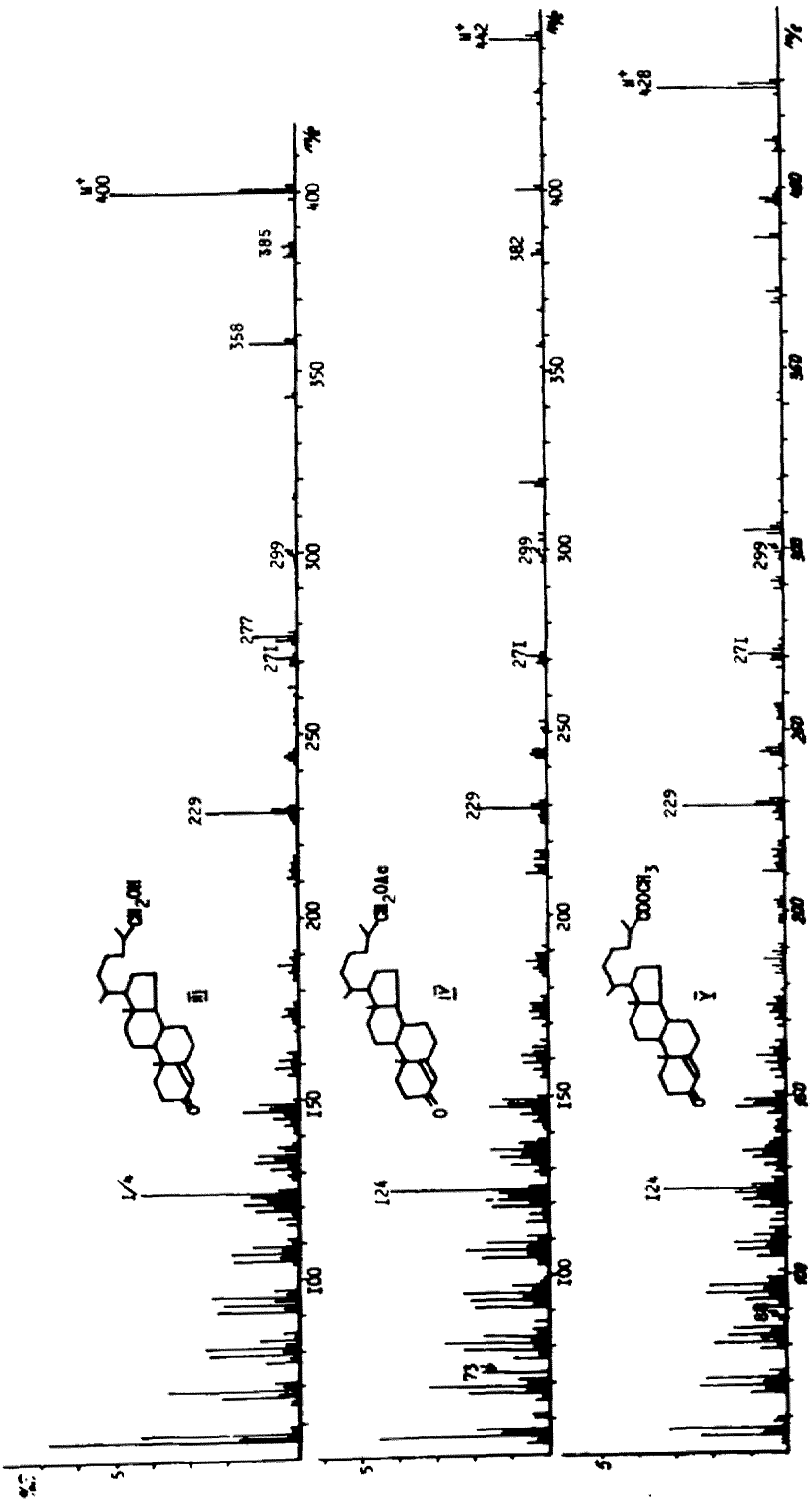
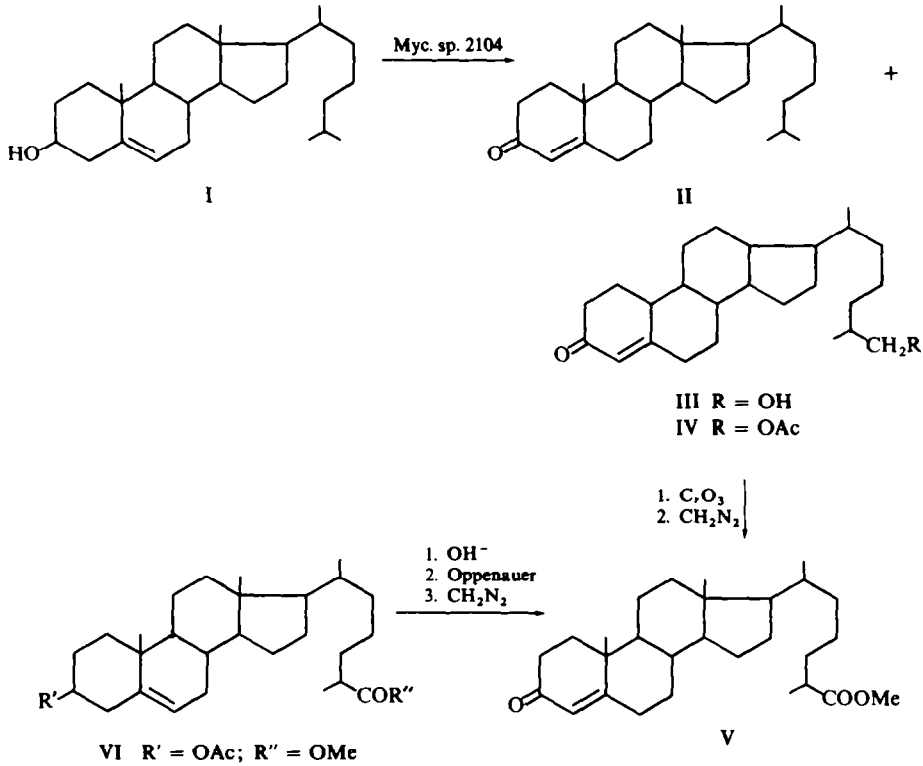


FIG. 1 Mass spectra of: (a) 27-hydroxy-cholest-4-en-3-one (III); (b) acetate of III (IV); (c) methyl 3-oxo-cholest-4-en-27-oate (V).

SCHEME I



I

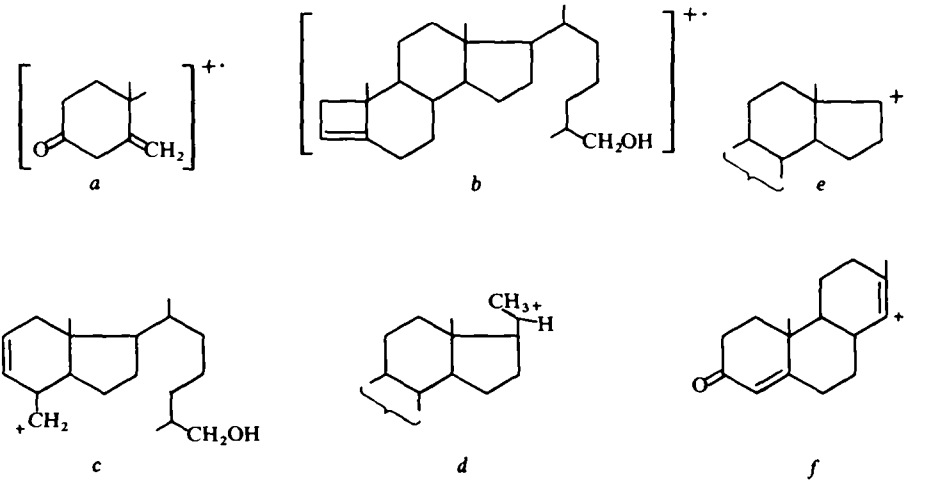
II

III R = OH
IV R = OAc

V

VI R' = OAc; R'' = OMe
VII R' = OH; R'' = OH

SCHEME II



a *b* *e*

c *d* *f*

OH group in the molecule of the latter. Thus, the analysis of the mass spectra of ketol III and its acetate IV shows that the OH group in III is at C-27 (or 26) or at C-21.

In the proton NMR spectrum of ketol III there are singlet signals due to the protons of the angular Me groups at C_{18} (0·70 ppm) and at C_{19} (1·18 ppm) as well as two partially overlapping doublets (J = 7 c/s) at 0·98 and at 1·07 ppm belonging to two Me groups, each coupled with the neighbouring methine proton. At 5·77 ppm there appears a resonance signal, with the intensity of one proton unit corresponding to the vinyl proton. The intensity of a doublet (J = 5 c/s) at 3·45 ppm equals two proton units. The chemical shift of this signal shows that the corresponding protons are located close to the oxygen atoms and interact with the OH protons according to the spin-spin mechanism with the constant J = 5 c/s. Thus, the NMR data prove the presence of the primary OH group in the molecule.

The primary character of the OH group and its location was also proved when the ketol III was oxidized according to Jones method into the corresponding acid which after treatment with diazomethane yielded methyl 3-oxo-cholest-4-one-27-oate (V).

The structure of the latter was proved by the mass spectral data. The spectrum of the ester V (Fig. 1c) reveals the ion peaks (ions *a*, and M-42) which are characteristic[3] of the cholest-4-en-3-one derivatives. The peak at *m/e* 88 is due to the $[CH_3CH_2COOCH_3]^{+\cdot}$ ion-radical which is of great importance because this type of fragmentation is peculiar to steroids with $COOCH_3$ group in position 27 (or 26).[4]

Finally, the structure of the methyl ester V was proved by its synthesis from methyl 3β-acetoxy-cholest-5-ene-27-oate (VI). The mass-spectrum and the chromatographic behaviour of methyl 3-oxo-cholest-4-ene-27-oate obtained independently from 3β-hydroxy-cholest-5-ene-27-oic acid (VII) and methyl ester V obtained from ketol III are identical. Thus III is 27-hydroxy-cholest-4-en-3-one, however, we have not yet established the stereochemistry at C-25.

When cholesterol I was fermented with *Mycobacterium sp.* 2104 the chromatographic analysis of the culture medium revealed first cholestenone (II) and then ketol III. The latter is also formed when the cholestenone itself is subjected to fermentation with the above culture. This proves that the culture of *Mycobacterium sp.* 2104 hydroxylates not cholesterol (I), but the cholestenone (II) formed from I during the fermentation.

Besides cholestenone (II) and 27-hydroxy-cholesten-4-en-3-one (III), the fermentation broth contained negligible amounts of unidentified UV absorbing polar substances.

According to preliminary data the formation of Δ^4-3-keto-group and the hydroxylation of the side chain occur when the β-sitosterol is fermented with *Mycobacterium sp.* 2104.

EXPERIMENTAL

The culture of *Mycobacterium sp.* 2104 was isolated from soil using the agar medium with cholic acid as the sole source of carbon.[1]

TLC was conducted on silica gel[5] and alumina (II–III activity) chromatoplates. Steroids on alumina were detected via UV light absorbtion and with I_2 vapour; on silica gel steroids were detected by means of Lugol or 2,4-dinitrophenylhydrazine reagents.

M.ps were taken on a "Boetius" microheater. IR spectra were taken with the Hilger H-800 spectrophotometer in the paste with vaseline oil. UV spectra were taken with SF-4 spectrophotometer. NMR spectra were recorded with JNMC-60 spectrometer in $CDCl_3$ using TMS as an internal standard. The mass spectra were measured on the Soviet commercial instrument MX-1303, furnished with a system allowing direct sample inlet into the ion source, at a temp 140–160° and ionizing energy 70 eV.

Fermentation of cholesterol with Mycobacterium sp. 2104. 10 l. of the nutrient medium containing 2 g $NaNO_3$, 1 g K_2HPO_4, 0·5 g KCl, 0·5 g $MgSO_4$, 0·01 g $FeSO_4$ and 10 g cholesterol dissolved in 150 ml EtOH (pH 7) were distributed equally among 500 ml flasks. After sterilization at 110–111° for 30 min, the flasks were inoculated with a culture of *Mycobacterium sp.* 2104 from the agar slant. The resulting mixture was fermented in the shaken flasks (200 rpm) at 28°.

After 48 hr fermentation, the contents of all flasks were combined and extracted by 4 × 4 l. $CHCl_3$, the extract was evaporated *in vacuo* to 1·5 l., dried ($MgSO_4$) and evaporated to dryness. The oily residue (5 g) after 4-fold preparative TLC on alumina (ether) yielded: (1) 3·15 g cholest-4-en-3-one, m.p. 78–80° (from alcohol) which showed no depression in m.p. upon admixture with the authentic sample; (2) 110 mg crude 27-hydroxy-cholest-4-en-3-one; (3) 110 mg cholesterol mixed with cholest-4-en-3-one; (4) 23 mg of the mixture of unidentified more polar substances absorbing in the UV light. The crude ketol III (110 mg) on TLC on alumina (ether) with subsequent crystallization gave rise to 30 mg of III m.p. 138–139·5°, $[\alpha]_D$ +95° ($CHCl_3$); $\lambda_{max}^{C_2H_5OH}$ 240 mμ; ν_{max} 1622, 1670 (Δ^4 −3 CO) 3460 (OH) cm^{-1}. NMR spectrum: 0·70 (18-CH_3); 1·18 (19-CH_3); 0·98, 1·07 (2CH—CH_3); 5·77 (vinyl proton), doublet 3·45 (proton neighbouring oxygen) ppm. R_s 0·63 (S-cholesterol; silica gel; hexane–AcOEt, 7:3).

The ketol gives a blue colour when the chromatogram is sprayed with Lugol reagent and a yellow colour when sprayed with 2,4-dinitrophenylhydrazine.

A soln of 10 mg of the crude ketol III in 1·5 ml pyridine was treated with 2 ml Ac_2O and allowed to stand overnight at room temp. 5 mg of 27-acetoxy-cholest-4-en-3-one was obtained, m.p. 81–83° (from ether); R_f 0·48 (silica gel; hexane–AcOEt 7:3).

Oxidation of 27-*hydroxycholest-4-en-3-one* (III). 2 mg of III was dissolved in 0·3 ml acetone and 150 mg Kiliani reagent (0·53 g CrO_3, 4 ml H_2O, 0·8 g conc H_2SO_4) was added at 0–5°. The reaction mixture was stirred at 22° for 20 min, then diluted with 2 ml water and extracted with 5 ml ether. The extract was washed with 2 ml water and 3 ml 0·5N KOH. The alkaline soln was acidified with 2 ml HCl (1:2) and extracted with ether. After the solvent was evaporated the crude acid (R_f 0·29) was treated with excess of an etheral soln of diazomethane. Ether was removed, the residue on TLC indicated the presence of V (R_f 0·42; silica gel; hexane–AcOEt 7:3).

Saponification of methyl 3β-*acetoxycholest-5-en-27-oate* (VI). The mixture of VI (10 mg) and KOH aq (2 ml; 0·03 g KOH, 0·5 ml H_2O and 1·5 ml MeOH) was refluxed for 1·5 hr. MeOH was evaporated *in vacuo* and the residue was diluted with 2 ml water and extracted with ether (3 × 3 ml). The extract was washed with 2 ml water. The aqueous alkaline soln was acidified with 2 ml HCl (1:2) and extracted with ether. On removal of ether, 6·5 mg of crude VII was isolated (R_f 0·23; silica gel; hexane–AcOEt 7:3).

Oppenauer oxidation of 3β-*hydroxycholest-5-en-27-oic acid* (VII). Crude VII (6·5 mg) was dissolved in a mixture of 4 ml benzene and 3 ml acetone. 3·5 ml of the solvent was distilled off and 0·5 g aluminium isopropoxide in 2·5 ml benzene was added. The reaction mixture was refluxed for 7 hr. After cooling, 7 ml water and 5 ml 10% H_2SO_4 was added. The aqueous layer was extracted with benzene. The benzene extract was dried over $MgSO_4$ and evaporated *in vacuo*. To the residue 2N KOH was added and extracted with ether. The aqueous layer was acidified with 1 ml HCl and extracted with ether. After the solvent was evaporated the residue (2 mg) was treated with excess of an etheral soln of diazomethane. The product obtained after the removal of ether was subjected to chromatography (silica gel; hexane–AcOEt 7:3). The chromatographic behaviour (R_f 0·42, mobility, colouration after spraying with Lugol reagent and 2,4-dinitrophenylhydrazine) as well as the mass spectral data of the reaction product proved it to be identical with methyl 3-oxo-cholest-4-en-27-oate obtained by oxidation of III.

Cholestenone and β-*sitosterol fermentations.* To 25 ml of the above mentioned nutrient medium, II (15 mg) in 1 ml EtOH was added; the mixture was sterilized at 110° for 30 min and inoculated with the culture of *Mycobacterium sp.* 2104. The mixture was fermented under the conditions described above. In 24 hr, the chromatographic test revealed the presence of III in the fermentation broth, R_s 0·63 (S-cholesterol; silica gel; hexane–AcOEt 7:3).

Under similar conditions, 15 mg β-sitosterol was fermented and 24 hr later, the chromatographic test showed the presence of β-sitostenone and a more polar UV-absorbing substance, R_s 0·63 (S-cholesterol; silica gel; hexane–AcOEt 7:3).

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