

**ISOLATION AND THE STRUCTURE OF THE PRODUCTS  
OF CHOLESTEROL BIODEGRADATION BY THE MUTANT  
*Mycobacterium* sp. CCM 3529**

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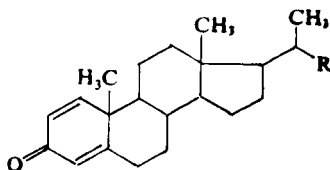
Received February 7th, 1984

During the biodegradation of cholesterol by the mutant *Mycobacterium* sp. CCM 3529 1,4-androstadiene-3,17-dione (*II*) is formed as the main product. The following compounds were identified as characteristic by-products: a mixture of methyl esters *IV* and *V* in a 1 : 3 ratio, 4-androstene-3,17-dione (*III*), 22-hydroxy derivative *I*, 17 $\beta$ -hydroxy-4-androsten-3-one (*XIII*) and 17 $\beta$ -hydroxy-1,4-androstadien-3-one (*XII*).

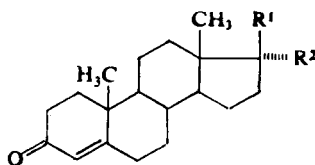
The mutant *Mycobacterium* sp. CCM 3528 affords 22-hydroxy-23,24-bisnor-1,4-choladien-3-one (*I*) as the main product of biodegradation of sterols, in addition to several by-products the structure of which has been described in the preceding paper<sup>1</sup>. In this paper we describe the structure of characteristic by-products of cholesterol biodegradation by the mutant *Mycobacterium* sp. CCM 3529 which affords 1,4-androstadiene-3,17-dione (*II*) (ref.<sup>2</sup>) as the main product.

The separation of the products was carried out by column chromatography on silica gel the course of which was monitored by thin-layer chromatography and gas chromatography. The following compounds were identified in the mixture by direct comparison with authentic samples: cholesterol (3.6%), 4-androstene-3,7-dione (*III*, 1.4%), 1,4-androstadiene-3,17-dione (*II*, 66.8%), 22-hydroxy-23,24-bisnor-1,4-choladien-3-one (*I*, 15.4%), 17 $\beta$ -hydroxy-4-androsten-3-one (*XIII*, 0.5%) and 17 $\beta$ -hydroxy-1,4-androstadien-3-one (*XII*, 2.8%). A further compound, methyl ester of 3-oxo-23,24-bisnor-1,4-choladienoic acid (*IV*), was obtained as a chromatographically inseparable mixture with a very similar substance, *V*, of the composition C<sub>23</sub>H<sub>30</sub>O<sub>3</sub> (mass spectrum). Alkaline hydrolysis of this mixture converted both its components to a mixture of acids (*VI* and *VII*); the mass spectrum displayed in addition to the base peak *m/z* 122 also peaks of molecular ions *m/z* 342 and 340. An analysis of the <sup>1</sup>H NMR, IR and UV spectra of the mixture of the esters *IV* and *V* indicated that another double bond in the admixture *V* is located in the position 17(20) (see the absence of the doublet of the C<sub>(21)</sub>-protons at 1.20 ppm and the presence of a new three-proton singlet at 1.95 ppm). This hypothesis was confirmed by the reduction of the 3-oxo group: in product *VIII* the UV absorption at 242 nm

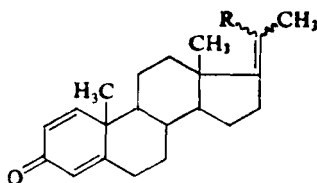
corresponds to the presence of an  $\alpha,\beta$ -unsaturated ester. The value of the maximum at 242 nm also displays a corresponding bathochromic shift with respect to the known *trans*-derivative<sup>3</sup> IX (222 nm), caused by the substitution with the methyl group in the  $\alpha$ -position of the conjugated system of compound VIII. An attempt at another synthesis of compound V from cyanohydrin X and unsaturated nitrile XI was unsuccessful.



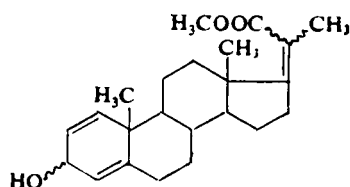
- I, R = CH<sub>2</sub>OH  
 IV, R = COOCH<sub>3</sub>  
 VI, R = COOH



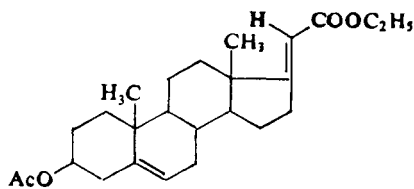
- II, R<sup>1</sup> = R<sup>2</sup> = O,  $\Delta^{1,4}$   
 III, R<sup>1</sup> = R<sup>2</sup> = O,  $\Delta^4$   
 XII, R<sup>1</sup> = OH, R<sup>2</sup> = H,  $\Delta^{1,4}$   
 XIII, R<sup>1</sup> = OH, R<sup>2</sup> = H,  $\Delta^4$



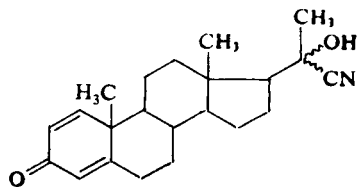
- V, R = COOCH<sub>3</sub>  
 VII, R = COOH  
 XI, R = CN



VIII



IX



X

## EXPERIMENTAL

The melting points were determined on a PHMK Wägetechnik Rapido microblock and they are not corrected. Optical rotation was measured in chloroform with a  $\pm 3^\circ$  accuracy. Thin-layer

chromatography was carried out on Silufol UV 254 Kavalier Votice in an unsaturated chamber with benzene-ether (1 : 1) as developing solvent. Column chromatography was carried out on Silica gel Silpearl (Kavalier, Votice), containing 10% of water. The  $^1\text{H}$  NMR spectra were measured on a BS 487C (80 MHz) Tesla instrument, in deuteriochloroform, using tetramethylsilane as internal reference. The chemical shifts are given in ppm. The mass spectra were measured on a MAT44S MCH 1320 instrument. Gas chromatography was carried out on a Chrom 5 instrument.

#### Isolation of 1,4-Androstadiene-3,17-dione (*II*)

The crude product of the biodegradation of 6.4 g of cholesterol (containing 8% of unreacted cholesterol and 29.5% of dione *II* according to gas chromatography) was dissolved in 200 ml of dichloromethane and the solution was washed with water, 5% hydrochloric acid, water, aqueous sodium carbonate and water. After drying over anhydrous sodium sulfate the solvent was evaporated, the residue (containing 11.8% of cholesterol and 47.6% of dione *II*) was dissolved in 20 ml of benzene-light petroleum (1 : 2) mixture and the solution was poured onto a silica gel column (20 g). Elution with 200 ml of the same solvent mixture gave non-polar admixtures and cholesterol. Product *II* was then eluted with 400 ml of benzene. After evaporation of benzene the crude product *II* (3.0 g, content of compound *II* 92%) was obtained, which was dissolved in 10 ml of acetone. This solution was treated with active charcoal and diluted with 30 ml of *n*-heptane. Yield, 2.4 g (51%) of 1,4-androstadiene-3,17-dione (*II*), m.p. 139–142°C. Literature<sup>4</sup> gives m.p. 137–146°C.

#### Isolation of the Cholesterol Biodegradation Products

The crude product of biodegradation of 2.91 g of cholesterol (weight 4.9 g) was dissolved in benzene and chromatographed on a silica gel column (250 g). Elution with benzene gave 0.106 g of cholesterol, followed by 0.160 g of a mixture of methyl esters *IV* and *V*. This mixture was crystallized from methanol. The product obtained had m.p. 135–142°C,  $[\alpha]_{\text{D}}^{20} + 22.9^\circ$  (*c* 0.18). UV spectrum:  $\lambda_{\text{max}}$  242 nm; the  $^1\text{H}$  NMR spectrum indicated that it is a mixture of two components. For the first component the following values were obtained: 7.08 (d, 1 H,  $J = 10.0$  Hz, 1-H); 6.24 (dd, 1 H,  $J = 10.0, 2.0$  Hz, 2-H); 6.08 (bs, 1 H, 4-H); 3.65 (s, 3 H,  $\text{COOCH}_3$ ); 1.28 (s, 3 H, 10- $\text{CH}_3$ ); 1.20 (d, 3 H, 20- $\text{CH}_3$ ); 0.80 (s, 3 H, 13- $\text{CH}_3$ ). The spectrum was identical with a spectrum of authentic methyl ester<sup>1</sup> *IV*. The second component had different values: 3.72 (s, 3 H,  $\text{COOCH}_3$ ); 1.95 (s, 3 H,  $\text{CH}_3\text{—C}=\text{C}$ ); 1.03 (s, 3 H, 13- $\text{CH}_3$ ). The values of the mass spectrum are given in the Theoretical. On alkaline hydrolysis in aqueous sodium hydroxide in methanol the product gave a chromatographically pure (TLC) substance with a free carboxyl group, m.p. 214–218°C,  $[\alpha]_{\text{D}}^{21} + 15^\circ$  (*c* 2.0), UV spectrum:  $\lambda_{\text{max}}$  241 nm.

Elution of the mixture with benzene-dichloromethane (1 : 4) gave 0.03 g of 4-androstene-3,17-dione (*III*), m.p. 168–172°C. On elution with pure dichloromethane 1.43 g of the main product *II* was obtained, m.p. 138–143°C. The mixture dichloromethane-acetone (8 : 1) eluted 0.01 g of 17 $\beta$ -hydroxy-4-androsten-3-one (*XIII*), m.p. 150–152°C, the same mixture in a 7 : 1 ratio eluted 0.38 g of 22 hydroxy derivative *I*, m.p. 180–183°C, while the 6 : 1 mixture eluted 0.06 g of 17 $\beta$ -hydroxy-1,4-androstadien-3-one (*XII*), m.p. 166–170°C. Compounds *III*, *II*, *XIII*, *I* and *XII* were identical with reference samples.

#### 2-Cyanohydrin *X*

1.5 g of 1,4-pregnadiene-3,20-dione were dissolved in 3 ml of acetone cyanohydrin by heating at 50°C, 0.3 ml of triethylamine were added and the mixture allowed to cool at room tempera-

ture. After 3 h standing the product separated (1.2 g) was filtered off under suction and crystallized from a mixture of dioxane and diisopropyl ether. The product had m.p. 166–175°C (decomp.).  $[\alpha]_D^{25} + 58^\circ$  (*c* 0.4, pyridine). For  $C_{22}H_{29}NO_2$  (339.5) calculated: 77.84% C, 8.61% H, 4.13% N; found: 77.57% C, 8.71% H, 3.99% N.

#### Nitrile XI

0.9 g of cyanohydrin *X* were heated with 0.5 ml of phosphorus oxychloride in 30 ml of pyridine under an inert atmosphere at 110–120°C for 1 h. The mixture was poured into dilute hydrochloric acid, extracted with dichloromethane and the extract washed with water till neutral. After drying over calcium chloride the solvent was evaporated to give 0.92 g of a residue which was chromatographed on 15 g of silica gel. Elution with benzene–dichloromethane (1 : 1) gave 0.36 g of chromatographically pure nitrile *XI* (according to TLC in benzene–dioxane–butyl acetate 88 : 10 : 2), which was crystallized from methanol. M.p. of the product was 192–198°C,  $[\alpha]_D^{25} + 137.8^\circ$  (*c* 0.5);  $^1H$  NMR spectrum: 7.05 (d, 1 H, 1-H); 6.21 (dd, 1 H, 2-H); 6.05 (bs, 1 H, 4-H); 1.80 (m, 3 H,  $CH_3-C=C$ ); 1.25 (s), 0.99 (s) (angular 10- $CH_3$  and 13- $CH_3$ ), UV spectrum:  $\lambda_{max}$  231 nm ( $\log \epsilon$  4.35). For  $C_{22}H_{27}NO$  (321.4) calculated: 82.20% C, 8.47% H, 4.36% N; found: 81.50% C, 8.65% H, 4.28% N.

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Translated by Ž. Procházka.