BIODEGRADATION OF CHOLESTEROL BY A MUTANT OF THE *Mycobacterium* SPECIES

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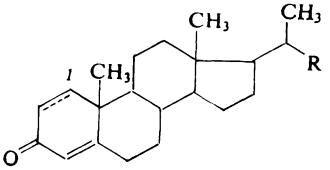
The biodegradation of cholesterol by the *Mycobacterium* mutant CCM 3528 gave rise to 22-hydroxy-23,24-bisnorchola-1,4-diene-3-one (*I*) as the main product. The identified by-products were 24-norchola-1,4-diene-3,22-dione (*IX*), androsta-1,4-diene-3,17-dione (*XV*) and their 4-unsaturated analogues, *X* and *XVI* respectively.

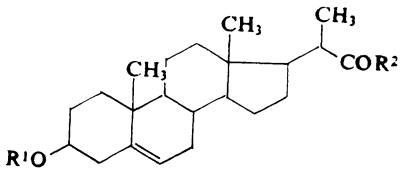
We have previously described[1] the biodegradation of cholesterol and some other sterols by new *Mycobacterium* mutants, *viz.* species CCM 3528 and CCM 3529, the former producing 22-hydroxy-23,24-bisnorchola-1,4-diene-3-one (*I*)* as the main product and the latter androsta-1,4-diene-3,17-dione (*XV*). The present paper demonstrates the structure of the 22-hydroxy derivative *I*, and describes the isolation and identification of the characteristic by-products of the biodegradation of cholesterol by the mutant *Mycobacterium* sp. 3528. The main product, isolated after the biotransformation by crystallization of the crude extract[1], had the molecular formula $C_{22}H_{32}O_2$. The UV and ^{1}H NMR spectra demonstrated the 1,4-diene-3-oxo grouping, the ^{1}H NMR spectrum also revealed the primary hydroxyl group. These findings are in accordance with the structure of the hydroxymethyl derivative *I*, *i.e.* the compounds arising from a partial degradation of the side chain of sterols. The structure was confirmed by comparing the physical constants with those of *I* first obtained by a microbiologic procedure in the biodegradation of sterols by *Mycobacterium* species NRRL B-3683 and NRRL B-3805, where it is formed as a by-product in the preparation of androsta-1,4-diene-3,17-dione[2] (*XV*). Thin-layer chromatography showed that the product *I* was always accompanied by a small admixture of a less polar compound, which was ascribed the structure of the 4-unsaturated 22-hydroxy derivative *II* (the hR_F values are given in Table I). Hydrogenation of *I* in the presence of a tris(triphenylphosphin)chlororhodium catalyst[3-5] afforded the 4-unsaturated derivative *II*, whose hR_F corresponded to the less polar admixture of *II* in the product *I* and whose physical constants[6] agreed with the assumed structure. The mixture of *I* and *II* could not be resolved by crystallization or column chromato-

* The names given in the literature are 20α-hydroxymethylpregna-1,4-diene-3-one[2] and (20*S*)-21-hydroxy-20-methyl-1,4-pregnadiene-3-one[17].

graphy into the individual compounds. Therefore, the crude *I*, isolated by chromatography of the mother liquor after crystallization of the bulk of *I* and accompanied by a small admixture of *II*, was converted into a mixture of acetates. Chromatography of this mixture, under the conditions specified in the isolation of the 22-ketones *IX* and *X*, gave a pure acetoxy deritative of the compound *II*. Alkaline hydrolysis of this acetate gave the 22-hydroxy derivative *II*, identical with the product *II* obtained by selective hydrogenation of the 1-double bond in the compound I.

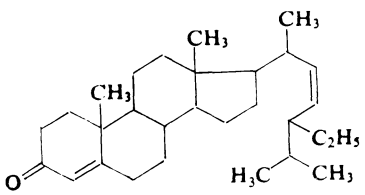
Oxidation of the 22-hydroxy derivative *I* by the Jones agent[7] gave the 1,4-unsaturated acid[8] *III*, which was converted by methylation with methanol under acid catalysis into its methyl ester[9] *IV*. An analogous oxidation of *II* led to acid *V*, which was converted in the same way into methyl ester[10] *VI*. The final proof of structures of the compounds *I* and *II*, including configuration on $C_{(20)}$, was obtained as follows: stigmasta-4,22-diene-3-one (*VII*) was ozonized into the well-known 20-aldehyde[10] *VIII*, whose oxidation by the Jones agent afforded the 4-unsaturated acid *V*, identical with the product obtained from the compound *II*. The methyl ester *VI* was dehydrogenated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to a product, which proved identical with the methyl ester *IV* obtained by the sequence *I* → *III* → *IV*.



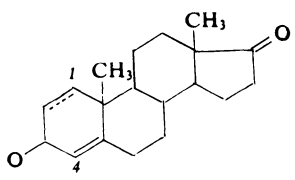


I, R = CH_2OH, $\Delta^{1,4}$
II, R = CH_2OH, Δ^4
III, R = COOH, $\Delta^{1,4}$
IV, R = $COOCH_3$, $\Delta^{1,4}$
V, R = COCH, Δ^4
VI, R = $COOCH_3$, Δ^4
VIII, R = CHO, Δ^4
IX, R = $COCH_3$, $\Delta^{1,4}$
X, R = $COCH_3$, Δ^4

XI, R^1 = CH_3CO, R^2 = OH
XII, R^1 = CH_3CO, R^2 = Cl
XIII, R^1 = CH_3CO, R^2 = CH_3
XIV, R^1 = H, R^2 = CH_3



VII



XV, $\Delta^{1,4}$
XVI, Δ^4

The mother liquors after separation of the bulk of *I* were resolved by thin-layer chromatography into a number of components, of which three pairs of compounds predominated. Judging by their hR_F values, these pairs consisted of the less polar 4-unsaturated and the more polar 1,4-unsaturated 3-oxo derivatives, the latter invariably prevailing. This assumption was corroborated by column chromatography. The most polar pair was found identical with the compounds *I* and *II*. The medium-polarity pair was identified by comparison with authentic samples as androsta-1,4-diene-3,17-dione (*XV*) and androst-4-ene-3,17-dione (*XVI*). The main lipophilic compound *IX* was demonstrated by elemental analysis and mass spectrometry to have the molecular formula $C_{23}H_{32}O_2$ (*m/z* 340). The UV and ^1H NMR spectra showed the presence of a 1,4-diene-3-oxo grouping, the ^1H NMR spectrum also detected a signal at 2·10 ppm ($COCH_3$). The presence of a methylketo group was corroborated even by other values of the mass spectrum. These findings are in keeping with the unusual structure of 24-norchola-1,4-diene-3,22-dione (*IX*), which was not previously found as a biodegradation product. The minor lipophilic component of the mother liquors is ascribed the structure of 24-norchol-4-ene-3,22-dione (*X*); this assignment is based on identical chromatographic behaviour of this compound and an authentic sample, obtained by selective hydrogenation of the 1-double bond in *IX*. The structures of compounds *IX* and *X* have also been proved by synthesis. Attempts at preparation of the 22-ketone *IX* by reaction of the ester *IV* with alkaline methylsulphinylmethide[13–15] have ended in failure. Consequently,

TABLE I
Values of hR_F on thin layer of silica gel[a]

Compound	S1	S2	S3	S4
Cholesterol	56	45	54	62
VI	56	45	58	67
IV	51	38	52	57
X	54	40	54	58
IX	49	31	48	46
XVI	45	26	45	41
XV	40	19	38	28
V	43	29	42	39
III	37	24	36	30
II	36	18	33	32
I	31	13	27	22

[a] S_1 benzene–acetone (70 : 30), S_2 benzene–ether (60 : 40), S_3 benzene–dioxan–butyl acetate (74 : 20 : 6), S_4 benzene–ether (50 : 50), double elution.

the following sequence of reactions was carried out: 3β-acetoxy-23,24-bisnorchol-5-ene acid (*XI*) was converted into chloride *XII*, whose reaction with dimethylcadmium[16] afforded 3β-acetoxy-24-norchol-5-ene-22-one[11] (*XIII*). Alkaline hydrolysis of *XIII* yielded the 3β-hydroxy derivative[11] *XIV*; the Oppenauer oxidation of the latter afforded the 3,22-dione[11,12] *X*, identical with the compound obtained by selective hydrogenation of 24-norchola-1,4-diene-3,22-dione (*IX*).

EXPERIMENTAL

The melting points, determined on a microblock PHMK Wägetechnik Rapido, are not corrected. The optical rotations were measured in chloroform with an accuracy of ±3°. Thin-layer chromatography was carried out on Silufol UV 254 Kavalier Votice in a saturated chamber, using systems given in Table I. The column chromatography was run on silica gel Kieselgel 60 (mesh 70–230), E. Merck, Darmstadt, containing 10% of water. The ^{1}H NMR spectra were measured using a spectrometer BS 487 C (80 MHz) Tesla (Czechoslovakia) in deuteriochloroform, with tetramethylsilane as internal standard. The chemical shifts are given in ppm. The mass spectrum was measured in an apparatus MAT 44S MCH 1320 (U.S.S.R.). The identities of the compounds were confirmed by mixed melting points and infrared spectra. The hydrogenation was carried out in thiophene-free benzene.

Working up of the Crude Extract Following the Biodegradation

The crude extract (3·9 g), obtained by biodegrading 2·17 g of cholesterol[1], was purified with activated carbon in methanol. The carbon was filtered off, the methanol was distilled off *in vacuo* and the residue was twice crystallized from ethyl acetate; yield 1·1 g of the 22-hydroxy derivative *I*: m.p. 180–182°C; $[\alpha]_D^{24}$ +28° *c* 0·9. UV spectrum: λ_{max} 247 nm (log ε 4·19). ^{1}H NMR spectrum: 7·02 (d, 1 H, *J* = 10·0 Hz, 1-H); 6·19 (mcd, 1 H, *J* = 10·0, 2·0 Hz, 2-H); 6·02 (bs, 1 H, 4-H), 3·45 (m, 2 H, —CH_2—OH); 2·12 (s, 1 H, OH); 1·20 (s, 3 H), 0·71 (s, 3 H) (angular CH_3); 1·00 (d, 3 H, *J* = 6·0 Hz, 20-CH_3). Reported[2] m.p. 181–183·5°C, $[\alpha]_D$ +28°.

The mother liquors were chromatographed on a column of silica gel (80 g). Elution with benzene and a mixture benzene–ethyl acetate 98 : 2 gave 0·21 g of the starting cholesterol; elution with benzene–ethyl acetate mixtures 90 : 10 and 88 : 12 afforded the crude 22-hydroxy derivative *I* (0·3 g), which was crystallized from ethyl acetate; yield 0·2 g of *I*, m.p. 181–183°C, $[\alpha]_D^{24}$ +27·5° (*c* 0·5). The total yield of *I* was 1·3 g (71%).

Isolation of By-products

After crystallization of the 22-hydroxy derivative *I*, the mother liquors left from several experiments were combined and taken to dryness. The dry residue (26·4 g) was dissolved in benzene (100 ml) and chromatographed on a column of silica gel (600 g). Elution with benzene and mixtures benzene–ethyl acetate 98 : 2 and 96 : 4 recovered 2·34 g of the unreacted cholesterol, elution with the same system in the ratio 98 : 10 gave 0·53 g of the 22-oxo derivative *X*, which was crystallized from methanol; yield 0·36 g, m.p. 203–205°C; $[\alpha]_D^{22}$ +52·5° (*c* 0·5). UV spectrum: λ_{max} 243 nm (log ε 4·21). ^{1}H NMR spectrum: 5·65 (bs, 1 H, 4-H); 2·10 (s, 3 H, $COCH_3$); 1·13 (s, 3 H), 0·74 (s, 3 H) (angular CH_3); 1·08 (d, 3 H, *J* = 6·0 Hz, 20-CH_3). Reported[11,12] m.p. 208°C, $[\alpha]_D$ +54°.

Elution with benzene–ethyl acetate 88 : 12 gave 1·56 g of the crude *IX*, which was twice crystallized from methanol; yield 1·21 g, m.p. 222–225°C; $[\alpha]_D^{22}$ −21° (*c* 0·8). UV spectrum: λ_{max}

246 nm (log ε 4·17). ^{1}H NMR spectrum: 7·02 (d, 1 H, J = 10·0 Hz, 1-H); 6·18 (mcd, J = 10·0, 2·0 Hz, 2-H); 6·02 (bs, 1 H, 4-H); 2·10 (s, 3 H, $COCH_3$); 1·23 (s, 3 H), 0·78 (s, 3 H) (angular CH_3); 1·11 (d, 3 H, J = 6·0 Hz, 20-CH_3). Mass spectrum: m/z 340 ($C_{23}H_{32}O_2$), 297 ($C_{21}H_{29}O$), 268 ($C_{19}H_{24}O$), 147 ($C_{10}H_{11}O$, $C_{11}H_{15}$), 122 ($C_8H_{10}O$). For $C_{23}H_{32}O_2$ (340·5) calculated: 81·13% C, 9·47% H; found: 80·91% C, 9·75% H.

Elution with benzene–ethyl acetate 87 : 13 afforded 0·41 g of androsta-1,4-diene-3,17-dione (*XV*), along with some androst-4-ene-3,17-dione (*XVI*). Repeated crystallization from acetone–hexane yielded 0·20 g of the diene *XV*, m.p. 134–138°C, $[\alpha]_D^{25}$ +112°C (*c* 0·7). Reported[17,18] m.p. 137–146°C, $[\alpha]_D$ +114°. The product was identical with an authentic sample.

Elution with benzene–ethyl acetate 86 : 14 gave 5·6 g of the 22-hydroxy derivative *I*, with an admixture of its analogue *II*. Crystallization from ethyl acetate yielded 4·1 g of *I*, m.p. 178 to 180°C, identical with the substance isolated by crystallization of the crude biodegradation extract.

22-Hydroxy-23,24-bisnorchol-4-ene-3-one (*II*)

A pre-hydrogenated suspension of the tris (triphenylphosphin) chlororhodium catalyst[3–5] (1·5 g) in benzene (30 ml) was mixed with a solution of *I* (1·5 g) in benzene (50 ml). The mixture was hydrogenated until no more hydrogen was taken up, chromatographed on a column of silica gel (50 g) and the product (*II*) was isolated by elution with benzene–dichloromethane 1 : 1. The solvent was distilled off *in vacuo* and the remaining catalyst was removed with activated carbon in hot methanol. The methanol was distilled off and the residue (1·22 g) was twice crystallized from ethyl acetate; yield 0·98 g (64%) of *II*, m.p. 135–138°C; $[\alpha]_D^{21}$ +94·5° (*c* 0·6). UV spectrum: λ_{max} 242 nm (log ε 4·18). Reported[6] m.p. 137–147°C, $[\alpha]_D$ +96°.

3-Oxo-23,24-bisnorchola-1,4-dienoic Acid (*III*)

To a stirred solution of *I* (2 g) in acetone (150 ml), cooled to +8°C, was added the Jones agent until a permanent orange colouring appeared. The stirring was continued at the same temperature for 30 min, the excess of the oxidant was decomposed with methanol and the mixture was concentrated *in vacuo* to a small volume. After an addition of water the product was collected on a filter, washed with water until the filtrate was neutral, and crystallized from ethyl acetate; yield 1·52 g (73%) of *III*, m.p. 237–239°C, $[\alpha]_D^{22}$ −18° (*c* 0·4). Reported[8] m.p. 234–237°C.

Methyl Ester of 3-Oxo-23,24-bisnorchola-1,4-dienoic Acid (*IV*)

a) A mixture of *III* (1·5 g), methanol (30 ml) and concentrated hydrochloric acid (1 ml) was boiled under a reflux condenser for 6 h, concentrated to a small volume and diluted with water. The product was taken into ether, the ethanol extract was washed with water until neutral, dried with magnesium sulphate, and the solvent was distilled off. The residue (1·4 g) was chromatographed on a column of silica gel (70 g). Elution with benzene–ethyl acetate 94 : 6 gave 1·3 g of the crude methyl ester *IV*, which was crystallized from methanol; yield 1·1 g (70%) of *IV*, m.p. 170–173°C, $[\alpha]_D^{23}$ −3·5° (*c* 1·6). Reported[9] m.p. 173–174°C.

b) A solution of the methyl ester *VI* (0·5 g) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0·45 g) in benzene (15 ml) was boiled for 24 h, any contact with the aerial moisture being excluded. After cooling, the mixture was filtered, the filtrate was washed with aqueous 2% potassium hydroxide and water, dried with magnesium sulphate, and the solvent was distilled off *in vacuo*. The residue (0·48 g) was chromatographed on silica gel (20 g). Elution with benzene–ethyl

acetate in the successive ratios 98 : 2, 96 : 4 and 95 : 5 afforded the product *IV*, which was crystallized from methanol; yield 0·28 g (56%) of *IV*, m.p. 169—173°C, identical with that obtained from the acid *III*.

3-Oxo-23,24-bisnorchol-4-enoic Acid (*V*)

a) The 22-hydroxy-derivative *II* (1 g) was oxidized with the Jones agent, analogously to the preparation of the acid *III*; yield 0·81 g (78%) of the acid *V*, m.p. 269—271°C, $[\alpha]_D^{23}$ +62° (*c* 0·4). Reported[10] m.p. 268—270°C, $[\alpha]_D$ +60°.

b) The aldehyde *VIII* (100 mg), obtained according to a described procedure[10] by ozonization of stigmasta-4,22-diene-3-one (*VII*), was oxidized by the Jones agent, analogously to the preparation of the acid *III*; yield 66 mg (63%) of the acid *V*, m.p. 266—269°C, identical with that obtained by procedure *a*).

Methyl Ester of 3-Oxo-23,24-bisnorchol-4-enoic Acid (*VI*)

Esterification of the acid *V* with methanol, using acid catalysis, was analogous to the preparation of the methyl ester *IV*; 1 g of *V* gave 0·63 g (60%) of *VI*, m.p. 176—179°C, $[\alpha]_D^{23}$ +67° (*c* 0·6). Reported[10] m.p. 178—184°C, $[\alpha]_D$ +70°.

24-Norchol-4-ene-3,22-dione(*X*)

a) One g of 3β-acetoxy-23,24-bisnorchol-5-enoic acid (*XI*) was converted into the dione *X* by a described sequence of reactions steps[11], the overall yield being 36%; m.p. 202—204°C, $[\alpha]_D^{21}$ +49° (*c* 0·4). Reported[11] m.p. 206—208°C, $[\alpha]_D$ +54°. The product was identical with that isolated from the mother liquors after the biodegradation.

b) One g of 24-norchola-1,4-diene-3,22-dione (*IX*) was selectively hydrogenated in the presence of the tris (triphenylphosphin) chlororhodium catalyst, analogously to the preparation of the 22-hydroxy derivative *II*; yield 0·63 g (61·5)% of the 3,22-dione *X*, m.p. 204—207°C $[\alpha]_D^{21}$ +53·5 (*c* 0·7), identical with the product obtained by procedure *a*).

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