MICROBIAL OXYGENATION OF 6β-HYDROXY-3α,5-CYCLO-5α-ANDROSTAN-17-ONE WITH *Rhizopus nigricans**

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Received August 20th, 1973

The transformation of 6β-hydroxy-3α,5-cyclo-5α-androstan-17-one with *Rhizopus nigricans* at pH close to 7 afforded a mixture of oxygenated products, of which the following were isolated and identified: the main product 6β,11α-dihydroxy-3α,5-cyclo-5α-androstan-17-one, further 2α,6β-dihydroxy-3α,5-cyclo-5α-androstan-17-one, 6β,7β-dihydroxy-3α,5-cyclo-5α-androstan-17-one, and 5-androstene-3β,11α,17β-triol which was evidently formed from 3α,5-cyclo-5α-androstane-6β,11α,17β-triol by isomerization during the isolation procedure.

For the proof of the structure of the main product of microbial hydroxylation of 3β-hydroxy-B-norandrost-5-en-17-one[1,2] by direct correlation we needed 3β,11α-dihydroxy-5-androsten-17-one (*I*) as starting compound. Its preparation was described in the patent literature only[3]. Our attempts at hydroxylation of dehydroepiandrosterone with various moulds did not bring positive results, because the hydroxylation took place as a rule in the position 7 (*cf.*[4]). None of the tested *Basidiomycetes*[5] gave the required product either. Vegetal tissues[6,7] also lacked the ability to hydroxylate in the position 11α. Therefore we applied the described procedure[8] for the preparation of 3β,11α-dihydroxy-5-pregnen-20-one *via* 6β,11α-dihydroxy-3α,5-cyclo-5α-pregnan-20-one to our case and we investigated using paper chromatography the conditions for the hydroxylation of 6β-hydroxy-3α,5-cyclo-5α-androstan-17-one (*II*) into the position 11α with some moulds of which *Rhizopus nigricans* was found most promising in this case as well. However, pH 7·5–8·0 given as optimum in the hydroxylation of 6β-hydroxy-3α,5-cyclo-5α-pregnan-20-one[8] was unsatisfactory in our case (lysis of the mycelium, low yields). Therefore we decreased it to pH 7·0 to 7·1 when the transformation went well. From the reaction mixture we isolated 4 products

* Part CLXIV in the series On Steroids; Part CLXIII: This Journal *39*, 603 (1974). Reported in part at the 9th International Congress for Microbiology, 24–30th July 1966, Moscow, and Conference on Progress in the Chemistry of Natural Substances, 16–18th October 1972, Smolenice, Czechoslovakia.

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of transformation (*A*, *B*, *C*, *D*). Their structure was determined mainly by the analysis of the PMR spectra of the substances and their derivatives and chemical correlation. Characteristic PMR parameters are listed in Table I.

The main product of transformation, substance *A* (yield about 30%) contained in addition to the hydroxy group in the position 6β another secondary hydroxy group. The splitting of the CH—O hydrogen atom of the newly introduced hydroxyl in the PMR spectrum (multiplet at 4·12 p.p.m. with three different vicinal coupling constants) indicated the presence of the structural fragment $—CH_2—CH(OH)—CH—$ and thus limited the possible positions of hydroxylation to $C_{(11)}$ and $C_{(15)}$. The width of the multiplet and the values of the vicinal coupling constants of the —CH—O— hydrogen ($W = 25·3$ Hz; $J_{vic} = 10·8$, 9·9 and 4·6 Hz), an analysis of the models, and the comparison with the described[9] characteristic shapes of the multiplets of variously hydroxylated Δ^4-3-oxosteroids led to the conclusion that the multiplet evidently belongs to the axial hydrogen in the position 11β and that the main product of hydroxylation has thus the structure of 6β,11α-diol *III*. This substance was submitted to acid catalysed isomerisation to unsaturated 3β-hydroxy derivative *I* which after Oppenauer oxidation afforded 11α-hydroxy-4-androsten-3,17-dione[10] which we prepared for correlation from 11α,17α,21-trihydroxy-4-pregnene-3,20--dione by side chain degradation with sodium bismuthate. Thus the supposed structure of the main product of transformation – 6β,11α-diol *III* – and of the unsaturated diol *I* formed by its isomerisation was confirmed chemically. Further confirmation of the structure of diol *I* was obtained from a comparison with an authentic sample prepared recently by direct hydroxylation of dehydroepiandrosterone with *Aspergillus ochraceus*[11].

From chromatographic mobility, elemental composition, IR and PMR spectra of product *B* (yield about 3%) and its acetate it was evident that it is also a monohydroxylated product with a different position of the newly introduced secondary hydroxyl. Chromatographic following of the rate of acetylation (acetic anhydride in pyridine at room temperature) and oxidation (chromium trioxide in pyridine) indicated appreciable differences in the reactivity of both hydroxyl groups. The comparison of the PMR spectra of the starting diol and the obtained products – monoacetate, diacetate, hydroxy diketone and triketone – showed that the newly introduced hydroxyl is more reactive during acetylation than the hindered 6β-hydroxyl. For the newly introduced hydroxyl the positions 1, 2, 11β, 12 and 15 were available. The hydrogen of the —CH—O-type at the newly introduced hydroxyl in the diol and its acetate appears in the spectrum as a doublet with a coupling constant 5·4 Hz. An analysis of the Dreiding models and the comparison with experimentally observed[9] characteristic multiplets of the —CH—O— protons in various positions of the steroid skeleton with unchanged annelation of the rings leads to the conclusion that only the hydroxy group localised on ring A may give

TABLE I

Chemical Shifts and Coupling Constants of PMR Spectra[a]

Compound	>CH—X (X = O, Cl)	$\diagdown\!\!\diagup$ CH_2	=CH—[b]	18-CH_3[c] 19-CH_3
I	C_3—H: 3·52 m, $W = 32$ Hz	—	5·44	0·90
	C_{11}—H: 4·12 m, $J_{11,12} = 10·7 + 5·0$, $J_{11,9} = 9·8$			1·19
IIa	C_6—H: 3·17 t, $J_{6,7} = 2·8 + 2·8$	0·19 dd	—	0·78
		0·40 t		0·95
IIb	C_6—H: 4·54 t, $J_{6,7} = 2·7 + 2·7$	0·47 m	—	0·91
				1·03
III	C_6—H: 3·27 t, $J_{6,7} = 2·8 + 2·8$	0·27 dd	—	0·94
	C_{11}—H: 4·00 m, $J_{11,9} = 9·9$, $J_{11,12} = 10·8 + 4·6$	0·61 t		1·21
IVa	C_2—H: 4·14 d, $J_{2,1} = 5·2 + \approx 0$, $J_{2,3} \approx 0$	0·31 t	—	0·86
	C_6—H: 3·33 t, $J_{6,7} = 2·6 + 2·6$	0·43 dd		1·01
IVb	C_2—H: 4·98 d, $J_{2,1} = 5·4 + \approx 0$, $J_{2,3} \approx 0$	0·43 t	—	0·90
	C_6—H: 3·33 t, $J_{6,7} = 2·6 + 2·6$	0·52 dd		1·06
IVc	C_2—H: 4·95 d, $J_{2,1} = 5·4 + \approx 0$, $J_{2,3} \approx 0$	0·29 t	—	0·80
	C_6—H: 4·45 t, $J_{6,7} = 2·6 + 2·6$	0·53 dd		0·91
Va	C_2—H: 3·64 m, $J_{2,1} = 11·2 + 4·4$, $J_{2,3} = 9·5$	—	5·35	0·82
	C_3—H: 3·28 m, $J_{3,4} = 9·7 + 7·0$, $J_{3,2} = 9·4$			1,00
Vb	C_2—H: 5·09 m, $J_{2,1} = 11·5 + 4·4$, $J_{2,3} = 10·0$	—	5·44	0·84
	C_3—H: 4·69 m, $J_{3,4} = 10·0 + 7·0$, $J_{3,2} = 10·0$			1·09
VI	C_6—H: 3·35 t, $J_{6,7} = 2·8 + 2·8$	[d]	—	0·91
				1·22
VII	—	[d]	—	0·89
				1·15
VIIIa	C_2—H + C_3—H: 3·68 m	—	5·44	0·86
				1·06
VIIIb	C_2—H: 5·08 m, $J_{2,1} = 11·6 + 4·7$, $J_{2,3} = 10·1$	—	5·48	0·89
	C_3—H: 3·73 m, $J_{3,4} = 10·1 + 7·1$, $J_{3,2} = 10·1$			1·15
IX	C_6—H: 3·23 d, $J_{6,7} = 3·7$	0·35 dd	—	0·93
	C_7—H: 3·47 dd, $J_{7,6} = 3·7$, $J_{7,8} = 9·8$	0·57 t		1·06
XI	C_3—H: 3·29 m, $W = 30$ Hz	—	5·28	0·67
	C_{11}—H: 3·80 m, $W = 25$ Hz			1·09
	C_{17}—H: 3·49 t, $J_{17,16} = 8 + 8$			

[a] The spectra were measured on a Varian HA-100 apparatus (100 MHz) in deuteriochloroform (with the exception of triol *XI* which was measured in a mixture of $CDCl_3 + CD_3SOCD_3$ 1 : 1) with tetramethylsilane (for unsaturated compounds) or with chloroform (for cyclopropyl-containing compounds) as internal references. In the latter case the chemical shifts are corrected to tetramethylsilane ($\delta_{CHCl_3} = 7·25$ p.p.m.); chemical shifts are given in the δ-scale (p.p.m.); coupling constants (obtained by first order analysis) are given in Hz. d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, W = width of the multiplet. [b] Broad doublets. [c] Singlets. [d] Signals of geminal cyclopropane protons are overlapped in the steroid envelope.

IIa; R = H
IIb; R = Ac
IIIa; R = H
IIIb; R = Ac
IVa; R¹ = R² = H
IVb; R¹ = Ac, R² = H
IVc; R¹ = R² = Ac
Va; R = H
Vb; R = Ac
I
VI
VII
VIIIa; R = H
VIIIb; R = Ac
IX
X
XI

rise to the observed doublet. For the hydroxylation in ring A the observed changes in chemical shifts of geminal cyclopropane hydrogens are also indicative (Table I). From the four possible positions of the secondary hydroxyl – *i.e.* 1α, 1β, 2α, 2β (others are excluded by the presence of the cyclopropane ring) – the positions 1β and 2β were eliminated on the basis of decoupling experiments. The selection between the two remaining possibilities (1α or 2α-OH) is very difficult to make on the basis of the PMR spectra. The vicinal coupling between C_3-H and $C_{2\beta}$-H and also between $C_{1\alpha}$-H and $C_{2\beta}$-H should be very close to zero according to the models (torsion angles about 90°), which enables the formation of the doublet of the —CH—O— hydrogen in 1α and 2α-hydroxy derivative. The selection between the two alternatives was done on the basis of acid catalysed isomerisation of the investigated diol. The unsaturated 3β-hydroxy derivative formed contains according to its IR spectrum an intramolecular hydrogen bond and PMR decoupling experiments proved the vicinal arrangement of both secondary hydroxyl groups in equatorial positions of the structural fragment —CH_2—CH(OH)—CH(OH)—CH_2—. Hence the product of isomerisation has the structure of the unsaturated 2α,3β-diol *Va* and its acetate structure *Vb*. Under the supposition that the acid catalysed isomerisation did not cause a change in the configuration of the introduced hydroxyl the product *B* must have the structure 2α,6β-diol *IVa*. The structures *IVb* and *IVc* belong to the above described products of its acetylation; the oxidation products have the structures *VI* and *VII* which are in accordance with the IR spectra. In an attempt at acid catalysed isomerisation of diol *IVa* in the presence of hydrochloric acid the unsaturated chlorinated alcohol *VIIIa* was formed which gave acetate *VIIIb* on acetylation. The structures of both substances were confirmed unambiguously by PMR spectra (Table I).

Product *C* was obtained in an approximately 2% yield. The appearance of a blue coloration on reaction with antimony trichloride (typical of allylic 7-hydroxy-5-enes) indicated that hydroxylation took place probably in position 7. This view was also supported by the relatively high mobility in paper chromatography, explicable by the decrease in polarity in consequence of an intramolecular hydrogen bond between the vicinal hydroxyls in the position 6 and 7. Acid catalysed isomerisation of the product gave a mixture in which the presence of a substance was demonstrated which had equal chromatographic properties as 7α and 7β-hydroxylated dehydroepiandrosterone (obtained on hydroxylation of dehydroepiandrosterone with *Rhizopus nigricans*). The position and the configuration of the newly introduced hydroxyl in product *C* was determined unambiguously from its PMR spectrum. The doublet and the doublet of doublets at 3·23 p.p.m. and 3·47 p.p.m. respectively may be assigned in the spectrum to the two —CH—O— hydrogen atoms of the secondary hydroxyl group. Decoupling experiments proved the vicinal arrangement of both hydroxyl groups in the structural fragment —C(—)—CH(OH)—CH(OH)—CH(—)—. As the com-

pound contains a structurally unchanged skeleton with a cyclopropane cycle and 6β-hydroxyl (the doublet at 3·2 p.p.m. belongs to $C_{(6)}$—H) the transformation product must have its second hydroxyl located in the position 7; from the IR spectrum (hydrogen bond) and from the magnitudes (values) of the coupling constants of the $C_{(7)}$ hydrogen (doublet of doublets at 3·47 p.p.m., $J_{6,7} = 3{\cdot}7$ Hz, $J_{7,8} = 9{\cdot}8$) it follows that the hydroxyl possesses the configuration 7β and hence product *C* has the structure of 6β,7β-diol *IX*.

The most polar product obtained, substance *D* (yield about 1%), did not contain a 17-oxo group according to the reaction with Zimmermann reagent and according to the IR spectra. The low chromatographic mobility and the results of elemental analysis indicated that in addition to the reduction of the oxo group a monohydroxylation also took place under formation of a triol. In view of the fact that the hydroxylation was carried out with *Rhizopus nigricans* and that the main product of hydroxylation was a substance hydroxylated in the position 11α, *i.e. III*, it was possible to infer that the product has the structure of 6β,11α,17β-triol *X*. However, as no cyclopropane ring could be proved in the substance (negative IR and PMR results) and the substance did not change on attempts at its acid isomerisation, it was assumed that the isomerisation of the primary product to the unsaturated 3β,11α,17β-triol had already taken place during the isolation procedure. This hypothesis was supported by the results of the PMR measurements which demonstrated the presence of the olefinic hydrogen $C_{(6)}$—H (broad doublet at 5·28 p.p.m.) and two —CH—O— hydrogens in the position 3β and 11α (multiplets at 3·29 p.p.m. or 3·80 p.p.m.), *i.e.* signals also occurring in the spectrum of diol *I*, and also another —CH—O— hydrogen (triplet at 3·49 p.p.m.) which is evidently due to the $C_{(17)}$ hydroxy group formed on reduction of the carbonyl group. The definite chemical proof of the structure was carried out by reduction of diol *I* with lithium aluminium hydride. The reaction product was identical with the isolated product of transformation to which, therefore, the structure of unsaturated 3β,11α,17β-triol *XI* should be attributed.

EXPERIMENTAL

The melting points were determined on a Kofler block and they are not corrected. Optical rotations were measured in chloroform unless otherwise stated. The infrared spectra were recorded on a UR-10 (Zeiss, Jena) spectrophotometer. For the PMR spectra see Table I. Silica gel (according to Pitra) was classified by sedimentation in water. For paper chromatography paper Whatman No 4 (saturated with vapours of 30% acetic acid) and a mixture of tetrachloromethane–acetic acid (50 : 1) was used, for thin-layer chromatography silica gel G (Merck) and ether, or chloroform–methanol (95 : 5), or ether–ethyl acetate (1 : 1). *Rhizopus nigricans* was obtained from the collection of microorganisms of the Research Institute of Pharmacy and Biochemistry, Prague.

Transformation of 6β-Hydroxy-3α,5-cyclo-5α-androstan-17-one (*IIa*) by *Rhizopus nigricans*

R. nigricans was cultivated under sterile conditions and under aeration and stirring in a medium (200 l) containing per 1 l of tap water 5 g of corn-steep liquor, 11 g of glucose, 0·4 g of K_2HPO_4, and 0·4 g $MgSO_4.7\,H_2O$, at 28—30°C for 24 hours. The mycelium was filtered under suction,

washed with a small amount of water and again submitted to suction. One half of the wet mycelium (10 kg) was suspended in 50 l of phosphate buffer of pH 7·0—7·1 (752·9 g Na_2HPO_4. .12 H_2O and 167·94 g KH_2PO_4). To the suspension a solution of 25 g of compound *IIa* in 500 ml of methanol was added in three portions (at one hour intervals) and the mixture was stirred and aerated for 20 hours. The mycelium was filtered off under suction and washed with a small amount of water. The filtrate was additioned with 10 kg of fresh mycelium and transformation was continued as above for another 20 hours. The mixture was filtered and the mycelium washed with water. Both mycelia were then washed on the filter with 30 l of chloroform and the chloroform filtrate was then used for extraction of the aqueous filtrate (buffer). The aqueous phase was extracted with another 50 l of chloroform. The combined extracts were dried and evaporated (34 g).

Isolation and Characterisation of the Transformation Products

The residue (34 g) was triturated with benzene (120 ml) and allowed to stand in refrigerator. The crystalline part of the material (6·85 g) was filtered off and the filtrate diluted with light petroleum. Thus another 6·2 g of precipitate were obtained. According to paper chromatography the combined fractions contained in addition to a smaller amount of the starting compound also a mixture of transformation products among which 4 prevailed: *A* the main product of transformation, R_F 0·37, *B* the more polar product of transformation, R_F 0·25, *C* the less polar product reacting with $SbCl_3$ in blue, R_F 0·71, *D* the most polar product of transformation, R_F 0·07.

The crude product (7·8 g) was chromatographed on 1·2 kg of silica gel with benzene (5 l) and ether. Ethereal eluates were analysed by thin-layer chromatography and combined appropriately. The first fractions containing predominantly product *A* were combined and after evaporation of solvent crystallised from acetone. Yield 4·6 g. After crystallisation from benzene the pure product *III* had m.p. 178—181°C, $[\alpha]_D^{20}$ +71° (*c* 2·18). For $C_{19}H_{28}O_3$ (304·4) calculated: 74·96% C, 9·27% H; found: 75·56% C, 9·20% H.

The mother liquors after crystallisation of product *A* were combined with subsequent fractions from the chromatography which contained the more polar product *B* and the mixture was rechromatographed. In this manner further 2·05 g of product *A* were obtained together with 390 mg of product *B* which was obtained on crystallisation from ethyl acetate and further chromatography in pure state (the mother liquors also contained product *C*). The pure product *B* (*IVa*, from benzene) had m.p. 185—188°C, $[\alpha]_D^{20}$ +114° (*c* 1·2). For $C_{19}H_{28}O_3$ (304·4) calculated: 74·96% C, 9·27% H; found: 75·02% C, 9·09% H. IR spectrum (cm^{-1}, CCl_4): 1 723 (five-membered cyclic CO), 1 408 (—CH_2—C=O), 3 030, 3 065 (cyclopropane), 3 623 (free OH).

From the last fractions of the above mentioned chromatographies a concentrate of product *D* (*XI*, 220 mg) was obtained which was purified by countercurrent distribution (methanol–water : : benzene–ether 65 : 35/85 : 15), chromatography on silica gel, and crystallisation from ethyl acetate and ethanol, m.p. 265—268°C; $[\alpha]_D^{20}$ −76° (*c* 0·265, chloroform–ethanol 1 : 1). For $C_{19}H_{30}O_3$ (306·4) calculated: 74·47% C, 9·87% H; found: 74·28% C, 9·68% H. IR spectrum (cm^{-1}, nujol); 3 240 (OH, strong band), absence of bands typical of cyclopropane ring and the carbonyl group.

Product *C* (*IX*) could not be separated chromatographically on a silica gel column (see above) from other transformation products. The crystalline crude mixture of products of transformation (7·74 g) was submitted to separation by countercurrent distribution which was repeated three times on a twenty-tube apparatus by the single withdrawal method. The first and the second countercurrent distribution was carried out using the system methanol–water/benzene–light petroleum (6 : 4/8 : 2) and the last one with the same mixture in a 7 : 3/6 : 4 ratio. A concentrate (400 mg) was thus obtained which was chromatographed on 200 g of silica gel with ether. Frac-

tions containing product *C* gave 240 mg of a substrate which when crystallised from acetone had m.p. 169–172°C, $[\alpha]_D^{20}$ +81° (*c* 0·45). For $C_{19}H_{28}O_3$ (304·4) calculated: 74·96% C, 9·27% H, 0·66% H act.; found: 74·87% C, 9·17% H, 0·49% H act.; IR spectrum (cm^{-1}, $CHCl_3$): 1 731 (five-membered cyclic CO), 3 620 (free OH), 3 565 (bound OH), 3 450 (assoc. OH), 3 060 (cyclopropane).

Acetates of the Products of Hydroxylation

Acetylations were carried out by the pyridine method in the cold.

Product *A* gave 6β,11α-diacetoxy-3α,5-cyclo-5α-androstan-17-one (*IIIb*), m.p. 160–161°C; $[\alpha]_D^{20}$ +32·5 (*c* 1·25). For $C_{23}H_{32}O_5$ (338·5) calculated: 71·10% C, 8·30% H; found: 71·23% C, 8·31% H.

Product *B* gave 2α,6β-diacetoxy-3α,5-cyclo-5α-androstan-17-one (*IVc*), m.p. 165–166°C; $[\alpha]_D^{20}$ +107° (*c* 1·09). For $C_{23}H_{32}O_5$ (388·5) calculated: 71·10% C, 8·30% H; found: 71·23% C, 8·22% H. IR spectrum (cm^{-1}, CCl_4): 1 740 (CO), 1 408 (—CH_2—C=O), 3 035, 3 070 (cyclopropane), no OH absorption.

Product *C* gave 6β,7β-diacetoxy-3α,5-cyclo-5α-androstan-17-one, amorphous. For $C_{23}H_{32}O_5$ (388·5) calculated: 71·10% C, 8·30% H; found: 70·45% C, 8·06% H.

Product *D* gave 5-androstene-3β,11α,17β-triol triacetate, amorphous; $[\alpha]_D^{20}$ −89° (*c* 2·08). For $C_{25}H_{36}O_6$ (432·5) calculated: 69·42% C, 8·39% H; found: 69·07% C, 8·35% H.

Isomerisation of Products *A*, *B*, and *C*

3β,11α-*Dihydroxy-5-androsten*-17-*one* (I): A solution of 6β,11α-dihydroxy-3α,5-cyclo-5α-androstan-17-one (*III*, 161 mg) in acetic acid (5 ml) was additioned with one drop of conc. sulfuric acid and refluxed on a water bath for one hour. The major part of the solvent was evaporated *in vacuo* and the residue diluted with 20 ml of ethyl acetate, the solution washed with water, sodium hydrogen carbonate, and water, and then dried and concentrated. The residue which contained the partially acetylated product was dissolved in 10 ml of methanol and to this solution 125 mg of K_2CO_3 dissolved in 1 ml of water were added and the mixture refluxed for 2·5 hours. After evaporation the residue was dissolved in a mixture of ether, ethyl acetate and water and the upper layer separated, washed with water and evaporated. The residue was crystallised from a mixture of benzene and light petroleum. Yield 115 mg, 208–210° (benzene–methanol), $[\alpha]_D^{20}$ −15·5° (*c* 2·07). For $C_{19}H_{28}O_3$ (304·4) calculated: 70·96% C, 9·27% H; found: 75·14% C, 9·33% H.

2α,3β-*Dihydroxy-5-androsten*-17-*one* (Va): A solution of 2α,6β-dihydroxy-3α,5-cyclo-5α-androstan-17-one (*IVa*, 209 mg) in 20 ml of acetone and 0·2 ml of 20% sulfuric acid was refluxed for one hour. After cooling, 0·5 ml of a saturated $NaHCO_3$ solution were added and the mixture evaporated. The residue was dissolved in ethyl acetate (20 ml) and washed with 5% $NaHCO_3$ and water and dried over sodium sulfate. The residue was crystallised from benzene–light petroleum. The pure product (190 mg) had a double melting point, 92–94°C and 168–174°C, $[\alpha]_D^{20}$ +6° (*c* 1·07). For $C_{19}H_{28}O_3$ (304·4) calculated: 74·96% C, 9·27% H; found: 75·19% C, 9·31% H. IR spectrum (cm^{-1}, $CHCl_3$): 1 408 (—CH—C=O), 1 734 (five-membered cyclic CO), 3 628 (free OH), 3 599 (bound OH).

3β,7-*Dihydroxy-5-androsten*-17-*one*: A solution of 6β,7β-dihydroxy-3α,5-cyclo-5α-androstan-17-one (*IX*, 2 mg) in 2 ml of acetone and 20 µl of 20% sulfuric acid was refluxed for 40 minutes. The reaction course was followed chromatographically on a thin layer of silica gel (chloroform–methanol 9 : 1). The spot of the starting substance was converted to two close spots which

according to R_F values and detection with $SbCl_3$ were identical with a mixture of 7-epimeric-3β,7-dihydroxy-5-androsten-17-ones prepared by microbiol hydroxylation of dehydroepiandrosterone with *R. nigricans*[4].

2α-Acetoxy-6β-hydroxy-3α,5-cyclo-5α-androstan-17-one (*IVb*) by Partial Acetylation of Product *B* (*IVa*)

2α,6β-Dihydroxy-3α,5-cyclo-5α-androstan-17-one (*IVa*, 156 mg) was dissolved in 3 ml of pyridine, additioned with 3 ml of acetic anhydride, and allowed to stand at room temperature for exactly 30 minutes. The mixture was poured onto ice and extracted with ether. The extract was washed with 3% HCl, 5% $NaHCO_3$ and water and dried over sodium sulfate and concentrated. The residue was chromatographed on a dry column of silica gel (15 g). The separation of diacetate was not complete. The fractions containing predominantly monoacetate were combined and crystallised twice from heptane; m.p. 173–176°C, $[\alpha]_D^{20}$ +109° (*c* 0·5). For $C_{21}H_{30}O_4$ (346·5) calculated: 72·80% C, 8·73% H; found: 72·09% C, 9·01% H. IR spectrum (cm^{-1}, CCl_4, $CHCl_3$): 1 730, 1 240 (OAc), 1 742 (five-membered cyclic CO), 1 408 (—CH_2—C=O), 3 035, 3 070 (cyclopropane), 3 500, 3 617 (OH).

2α-Hydroxy-3β-chloro-5-androsten-17-one (*VIIIa*)

To a solution of substance *IVa* (97·3 mg) in 10 ml of acetone 60 μl of conc. HCl were added and the mixture refluxed for 30 minutes. The solvent was distilled off in vacuo and the residue dissolved in 2 ml of methanol and diluted with 5 ml of water. A product (60 mg) crystallised out which after further crystallisation from heptane and ethyl acetate melted at 154–155°C and then at 164–165°C, $[\alpha]_D^{20}$ +1·8° (*c* 0·63). For $C_{19}H_{27}ClO_2$ (332·9) calculated: 70·67% C, 8·42% H, 10·98% Cl; found: 70·86% C, 8·50% H, 10·76% Cl. IR spectrum (cm^{-1}, CCl_4): 1 742 (five-membered cyclic CO), 1 408 (—CH_2—C=O), 1 671, 3 040 (C=C), 3 470, 3 590 (OH). Acetate *VIIIb* was prepared by the pyridine method in the cold; m.p. 209–210°C, $[\alpha]_D^{20}$ −75° (*c* 0·87).

2α,3β-Diacetoxy-5-androsten-17-one (*Vb*)

2α,3β-Dihydroxy-5-androsten-17-one (*Va*, 30 mg) was dissolved on 0·5 ml of pyridine and 0·5 ml of acetic anhydride and the mixture allowed to stand overnight. The solvents were evaporated *in vacuo* and the residue crystallised from aqueous methanol and then light petroleum. Yield, 28 mg, m.p. 138–139·5°C, $[\alpha]_D^{20}$ −59° (*c* 0·84). For $C_{23}H_{32}O_5$ (388·5) calculated: 71·10% C, 8·30% H; found: 71·23% C, 8·22% H. IR spectrum (cm^{-1}, CCl_4): 1 740 (C=O), 1 408 (—CH_2—C=O), 1 674, 3 040 (C=C), no OH.

11α-Hydroxy-4-androsten-3,17-dione (*XII*)

A) A solution of 3β,11α-dihydroxy-5-androsten-17-one (*I*, 83 mg) in 4 ml of toluene and 1·5 ml cyclohexanone was distilled. After 1 ml of the solvent was distilled off 0·5 ml of an aluminium 2-propoxide (100 mg) solution in toluene was added to it and the distillation continued slowly for 20 minutes (1·5 ml of distillate). After cooling the mixture was poured onto ice and acidified with 5 ml 4% HCl. The mixture was extracted with ether and the extract washed with water, sodium hydrogen carbonate and water and the extract concentrated. The residue was crystallised from ethyl acetate (39 mg), m.p. 225–226°C; $[\alpha]_D^{20}$ +164° (*c* 0·95) (lit.[11] gives 226–227°C and $[\alpha]_D^{20}$ +164°). For $C_{19}H_{26}O_3$ (302·4) calculated: 75·46% C, 8·67% H; found: 75·08% C, 8·67% H.

B) A solution of 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (200 mg) in 3 ml of acetic acid was diluted with 3 ml of water and 2·5 g of $NaBiO_3$ were added to it and the mixture shaken in darkness for 2 hours. The mixture was diluted with 40 ml of ethyl acetate, shaken and decanted. The solid residue was extracted several times with ethyl acetate. The precipitate was filtered off and washed on the filter with 20 ml of ethyl acetate. The filtrate was concentrated to 25 ml volume, diluted with 25 ml of ether and washed with sodium hydrogen carbonate solution and water. The organic layer was dried over sodium sulfate and evaporated. The residue was crystallised from ethyl acetate, yield 90 mg; m.p. 225–227°C, $[\alpha]_D^{20}$ +166° (*c* 0·90). IR spectra of both products were identical.

5-Androstene-3β,11α,17β-triol (*XI*)

To a solution of 3β,11α-dihydroxy-5-androsten-17-one (60 mg) in ether (6 ml) and tetrahydrofuran (2 ml) lithium aluminium hydride (103 mg) was added in several portions. The mixture was allowed to stand overnight, then decomposed with water and extracted with 5 ml of a 20% sulfuric acid solution. An insoluble substance precipitated out which was filtered off and dried (38·7 mg), m.p. 240–261°C. From the ethereal extract another 20 ml of a substance were obtained which was identical with the precipitated product, which when crystallised from ethyl acetate had m.p. 240–261°C. The combined fractions were recrystallised from ethyl acetate, affording 49 mg of a product melting at 265–267°C, $[\alpha]_D^{20}$ −76° (chloroform–ethanol 1 : 1, *c* 0·41). The substance melted undepressed on admixture of product *D*. The colour test with $SbCl_3$, the R_F values and the IR spectra were also identical.

3α,5-Cyclo-5α-androstane-2,6,17-trione (*VII*)

A solution of 2α, 6β-dihydroxy-3α,5-cyclo-5α-androstan-17-one (*IVa*, 203 mg) in 20 ml of pyridine was added at once into a solution of 1 g of chromium trioxide in 10 ml of pyridine at 0°C. The mixture was allowed to stand at room temperature for 20 hours and then decomposed by pouring it into 250 ml of a saturated $NaHCO_3$ solution which was extracted three times with 150 ml of ether. The extract was washed with dilute hydrochloric acid, water, sodium hydrogen carbonate and water and dried over sodium sulfate. After filtration the solution was evaporated (247 mg) and crystallised from a mixture of isopropyl ether and ethanol (3 : 2) and then from ethanol (10 ml). Yield 204 mg, m.p. 231–233°C (sublimates); $[\alpha]_D^{20}$ +69° (*c* 1·9). For $C_{19}H_{24}O_3$ (300·4) calculated: 75·97% C, 8·05% H; found: 75·52% C, 7·88% H. IR spectrum (cm^{-1}, $CHCl_3$): 1 700 (six-membered cyclic CO), 1 735 (strong band of a five-membered cyclic CO).

6β-Hydroxy-3α,5-cyclo-5α-androstane-2,17-dione (*VI*)

A solution of substance *IVa* (135 mg) in 4 ml of pyridine was added at once to a solution of 0·35 g of CrO_3 in 5 ml of pyridine at 0°C. The mixture was allowed to stand at 0°C for 95 minutes and poured into 150 ml of 10% $NaHCO_3$. The solution was extracted four times with 150 ml of ether, the extract washed with dilute hydrochloric acid, water, hydrogen carbonate, and water, then dried over sodium sulfate and evaporated to dryness. The residue (104 mg) was chromatographed on a dry column of silica gel (30 g) with a light petroleum–chloroform mixture 1 : 1. The fractions were analysed chromatographically on thin layers. The product (free of either the starting compound or triketone *VII*) was not individual and therefore it was rechromatographed first on a column and then on a preparative plate with a 1 mm strong layer of silica gel G, using a mixture of chloroform and methanol 100 : 2 for development. The substance (26 mg) of higher R_F value, which gave a dark spot on detection with conc. sulfuric acid and heating (the weaker close zone

reacted in yellow), was extracted with methanol, the methanolic extract was evaporated, re-extracted with chloroform, the extract evaporated, and the residue crystallised from heptane–ethyl acetate. Yield 20 mg, m.p. 201–203°C, $[\alpha]_D^{20}$ +119° (*c* 0·71). For $C_{19}H_{26}O_3$ (302·4) calculated: 75·46% C, 8·67% H; found: 73·05% C, 8·44% H. IR spectrum (cm^{-1}, $CHCl_3$:) 1 405 (—CO—CH_2—), 1 733 and 1 720 sh (two five-membered cyclic CO groups), 3 612 (OH).

For the cultivation of the microorganism and the large scale transformation we thank Dr A. Čapek and Mr J. Tůma, Research Institute of Pharmacy and Biochemistry, Prague, and Dr A. Břečka and Dr E. Toscaniová, Antibiotics Research Institute, Roztoky near Prague. For the measurement and the interpretation of the IR spectra we thank Dr J. Smolíková and Dr S. Vašíčková, Department of Molecular Spectroscopy of our Institute, and for the measurement and the interpretation of some PMR spectra our thanks are due to Dr Z. Samek of the same Institute. We are grateful to Mrs A. Ebelová for technical assistance. We also thank Dr K. Kieslich, the Main Laboratory of Schering AG, Berlin, for the kind supply of the sample of 6β,11α-dihydroxy-3α,5-cyclo-5α-androstan-17-one and Dr A. Čapek, Research Institute of Pharmacy and Biochemistry, Prague, for a sample of epi-hydrocortisone.

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Translated by Ž. Procházka.