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REDUCTION OF 20-OXOSTEROIDS TO 20α-ALCOHOLS BY MEANS OF ACTINOMYCES ROSEOCHROMOGENUS

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It is known that many microorganisms reduce 20-oxosteroids to 20β-hydroxy compounds [1,2]. The formation of 20α-hydroxysteroids by microorganisms has been observed in only a few cases [3-7].

In an investigation of the conversions of steroids by actinomycetes, we found that a culture of A. roseochromogenus ATCC 3347 reduces some 17-oxygen containing pregnenes to the 20α-hydroxy compounds (the capacity of actinomycetes for performing this reaction was previously unknown). The most characteristic reaction for A. roseochromogenus is 16α-hydroxylation. This organism is capable of performing 2β-hydroxylation, 1,2-dehydrogenation, and the reduction of 20-oxosteroids to the 20β-hydroxy compounds (see Table 1, VII) [2].

The fermentation of 17α-hydroxyprogesterone (I) with a culture of A. roseochromogenus ATCC 3347 gave a substance (II) with the composition $C_{21}H_{32}O_3$. Its UV spectrum has a maximum at 241 mμ, which is characteristic for a Δ^4-3-oxo grouping. The IR spectrum contains the band of a hydroxy group but not that of a 20-oxo group.

When substance II was subjected to thin-layer chromatography, it was found that its R_f value was lower than that for 17α,20β-dihydroxypregn-4-en-3-one, obtained from 17α-hydroxyprogesterone by Norymberski and Woods' method [8]. The chromatographic mobilities of the 20α-hydroxysteroids are somewhat lower than or equal to those of the corresponding 20β-epimers [9,10].

On this basis, substance II was assigned the structure of 17α,20α-dihydroxypregn-4-en-3-one. Its melting point and specific rotation are close to the figures given by Neher and Wettstein [11]. The acetylation of II led to the 20-acetate (III), the physical constants of which agree with those given in the literature [11]. The α-configuration of the 20-hydroxy group is confirmed by a comparison of the molecular optical rotation of II and its acetate III. The increment in the molecular rotation of the 20α-acetoxy group has a negative value [12,13]. In our case, $M^{Ac-OH} = -75°$.

The incubation of 11α,17α-dihydroxyprogesterone (IV) gave compound V, the molecular weight of which corresponds to a dihydro derivative of the dihydroxydiketone IV.

The IR spectrum of the trihydroxyketone V contains absorption bands characteristic for hydroxy groups and for an α,β-unsaturated ketone but does not contain the absorption band of a 20-oxo group and differs from the IR spectrum of 11α,17α,20β-trihydroxypregn-4-en-3-one, obtained by the reduction of the dihydroxyketone (V) [14] with sodium borohydride [8]. The melting points of V and of 11α,17α,20β-trihydroxypregn-4-en-3-one are different (274-276° C and 215-217.5° C, respectively). On this basis, substance V was ascribed the structure of 11α,17α,20β-trihydroxypregn-4-en-3-one.

When 16α,17-epoxyprogesterone (VI) was fermented, two substances—VII and IX—having lower chromatographic mobilities than VI were isolated. The IR spectra of substance VII and its monoacetate (VIII) showed no absorption band characteristic of an α,β-unsaturated ketone. This shows that fermentation leads to the reduction both of the 3-oxo group and of the double bond, which is confirmed by the absence of the signal of a vinyl proton from the NMR spectrum. Such a reduction of an α,β-unsaturated ketone may lead to the formation of one of four structures: 3β-hydroxy-5α-, 3β-hydroxy-5β-, 3α-hydroxy-5α-, and 3α-hydroxy-5β-pregnanes. The selection of the structure of compound VIII was made on the basis of a calculation (by Zurcher's method [15]) of the chemical shifts of the 18- and 19-methyl groups of each of the four possible isomers and a comparison of the calculated figures with the values obtained experimentally for the acetate VIII (Table 1).

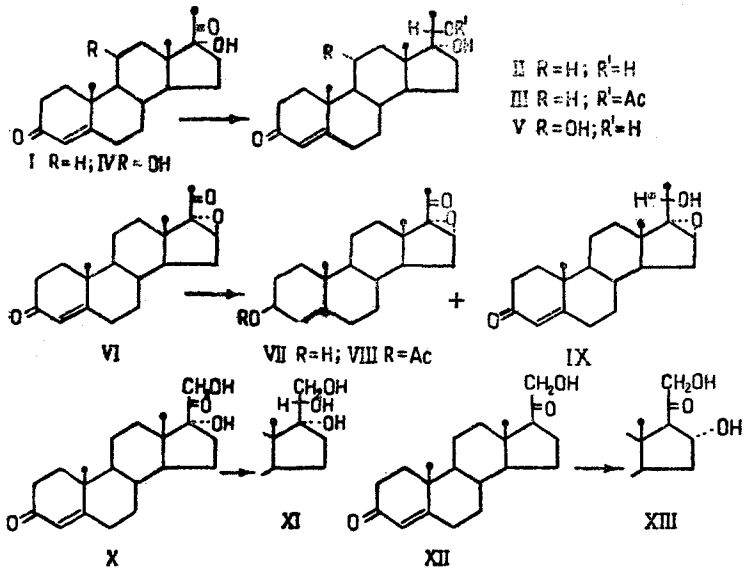
It can be seen from Table 1 that substance VIII can be ascribed the structure of the acetate of 16α,17-epoxy-3β-hydroxy-5α-pregnan-20-one. The structure of a 3α-acetoxy-5α-pregnane is excluded since the NMR spectrum of VIII has a broadened signal at 4.65 ppm from the axial proton at C_3. The chemical shifts of the angular methyl groups of the acetate VIII agree with the results given for this substance by Japanese workers [16]. The results of a comparison of the second substance that we isolated (IX) with 16α,17-epoxy-20α-hydroxypregn-4-en-3-one, obtained previously by Suvorov et al. [6], showed that the two substances are identical with respect to their IR spectra, chromatographic behavior, and melting point. Consequently, substance IX was ascribed the structure of 16α,17-epoxy-20α-hydroxypregn-4-en-3-one. The R_f value of substance IX is somewhat lower than that of 16α,17-epoxy-20β-hydroxypregn-4-en-3-one obtained by the method of Norymberski and Woods [8].

The fermentation of cortexolone (X) led to the formation of a more polar substance (XI) in low yield.

Methyl group	Calculated				Experimental result	
	3β-OAc-5α-H	3β-OAc-5β-H	3α-OAc-5α-H	3α-OAc-5β-H	(VIII)	16α,17-epoxy 3β-hydroxy-5α-pregnan-20-one acetate [16]
18—CH_3	1.03	1.03	1.04	1.03	1.02	1.02
19—CH_3	0.84	0.98	0.82	0.95	0.83	0.83

When a chromatogram of substance XI was treated with isonicotinic acid hydrazide [17], it was found that XI lacked a double bond in the 1,2-position. The chromatographic behavior and IR spectrum of XI permit the conclusion that this substance is 17α,20α,21-trihydroxypregn-4-en-3-one.

After the fermentation of 11-deoxycorticosterone (XII), a substance (XIII) was obtained the chromatographic behavior of which was identical with that of an authentic sample of 16α,21-dihydroxypregn-4-ene-3,20-dione.



It was interesting to compare the action of intact cells of A. roseochromogenus with the action of a cell-free preparation obtained from it. For this purpose, a culture grown in the presence of 11α-hydroxyprogesterone (inductor) was broken down by ultrasonics. The homogenate was centrifuged and the 6750 g supernatant was used as the source of enzymes. The incubation of 17α-hydroxyprogesterone (I), 11α,17α-dihydroxyprogesterone (VI), and 16α,17-epoxyprogesterone (VI) with the cell-free extract gave the corresponding 20α-alcohols II, V, and IX. As in the case of a culture of the actinomycete, the incubation of 16α,17-epoxyprogesterone (VI) with the cell-free preparation yielded 16α,17α-epoxy-3β-hydroxy-5α-pregnan-20-one (VII).

The incubation of cortexolone (X) with the 6750 g supernatant led to no detectable formation of XI.

The supernatant obtained by centrifuging the homogenate at 90,000 g reduced 17α-hydroxyprogesterone (I) to the dihydroxyketone II.

The fermentation of a culture of A. roseochromogenus ATCC 3347 with steroids showed that the introduction of an oxygen function into the 17α position of the progesterone molecule prevents the 16α-hydroxylation of the steroid. Moreover, steroids with a 17α oxygen function are reduced by a culture of A. roseochromogenus, forming the corresponding 20α-alcohols. This relationship is preserved in incubation with a cell-free preparation and, consequently, is due to the nature of the enzyme and is not affected by such factors as thermal permeability and the induction of enzyme synthesis.

According to Engel and Rakhit [18], the side chian of a pregnane steroid may be present in one of four preferred conformations, two of which—the so-called A-type conformations—promote the formation of 20α-alcohols on reduction with metal hydrides, according to Cram's rule, while the other two—B-type conformations—permit the formation of 20β-alcohols.

For 17α-hydroxyprogesterone (I), cortexolone (X), 11α,17α-dihydroxyprogesterone (IV), and 16α,17-epoxyprogesterone (VI), a conformation of the side chain of type B is predominant, i.e., the conformation favoring the formation of the β-alcohols. Consequently, to obtain the α-alcohols it is necessary first to convert the B conformation into a A conformation. It may be assumed that such a conversion takes place at the stage of the formation of an enzyme-substrate complex, the 17α oxygen function (hydroxy group, epoxide) ensuring the binding with the enzyme and the change in the

conformation of the side chain. In the case of 11α,17α-dihydroxyprogesterone (IV), the yield of dihydro 20α derivative (V) is considerably lower than the yield of the corresponding alcohol in the fermentation of 17α-hydroxyprogesterone (I). This shows the inhibiting influence of an 11α-hydroxy group on the reduction of a 20-ketone. The inhibiting influence of a 21-hydroxy group is still more pronounced: on fermentation with cortexolone, the 20α-alcohol is formed in trace amounts. These results are similar to those obtained by Chang and Idler [4], who showed that a 17α-hydroxy group accelerates and 11α- and 21-hydroxy groups retard the α-reduction of 20-oxopregnanes by means of a completely different microorganism—Rhodotorula longissima. Apparently, a 21-hydroxy group also inhibits 16α-hydroxylation— on fermentation with deoxycorticosterone, 16α,21-dihydroxyprogesterone is formed in very low yield, while on fermentation with progesterone 16α-hydroxyprogesterone is formed in good yield.

The formation of 16α,17-epoxy-3β-hydroxy-5α-pregnan-20-one (VII) from 16α,17-epoxyprogesterone (VI) with the aid of A. roseochromogenus is the third case of the conversion of Δ^4-3-oxo- into 3β-hydroxy-5α-steroids by actinomycetes. This reaction has been described previously by Vischer and Wettstein [19] for Streptomyces griseus and by Kondo et al. [20] for S. aureus.

Experimental

The melting points were determined on a "Boetius" micro heating stage, and the specific rotation were measured in chloroform. Before analysis the samples were dried at 100° C in vacuum (0.1 mm Hg) for 6 hr.

The IR spectra were taken on a Hilger H-800 spectrophotometer (mull with paraffin oil), the UV spectra on an SF-4 spectrophotometer (ethanol), the NMR spectra on a JNM-4-H100 instrument with a working frequency of 100 MHz (solvent $CDCl_3$; standard, hexamethyldisiloxane), and the mass spectra on a MKh-1303 instrument.

Growth of the seed material. One hundred milliliters of a nutrient medium containing 10 g of starch, 2 g of $(NH_4)_2SO_4$, 1 g of $MgSO_4$, 1 g of NaCl, 1 g of K_2HPO_4, and 3 g of $CaCO_3$ per *l* of tap water (pH 7.0) was included with the air-dry mycelium of Actinomyces roseochromogenus from an agar slope and the culture was grown under aerobic conditions at 28° C in a rotary shaking machine (200 rpm) for 72 hr.

Fermentation of steroids with A. roseochromogenus. Flasks containing 100 ml of the medium described above were inoculated with the seed material, 10 ml of the culture being added to each flask. The cultures were grown for 1 day at 28° C on a rotary shaking machine (200 rpm) under aerobic conditions. Then a solution of a steroid in 0.5 ml of ethanol was added to each flask. After the end of fermentation, the mycelium was filtered off and washed with hot water, and the filtrate and wash-waters were combined and extracted with methylene chloride. The extract was dried over magnesium sulfate and evaporated to dryness. The fermentation products were isolated from the residue by chromatography and crystallization.

Chromatography. Plates were prepared with a fixed layer of type KSK silica gel by a published method [21]. Several system of solvents were used: 1) ether; 2) ethyl acetate—acetone (4 : 1); 3) benzene—acetone (4 : 1); 4) ethyl acetate; and 5) benzene—acetone (2 : 1). Chromatography on silica gel gave the following R_f values (commercial alumina was used for chromatography on alumina):

Substance	R_f, system	Coloration with Lugol's solution
17α,20α-Dihydroxypregn-4-en-3-one (II)	0.23 (1)	Blue
17α,20β-Dihydroxypregn-4-en-3-one	0.30 (1)	Yellow
11α,17α,20α-Trihydroxypregn-4-en-3-one (V)	0.29 (2)	White
11α,17α,20β-Trihydroxypregn-4-en-3-one	0.29 (2)	White
16α,17-Epoxy-20α-hydroxypregn-4-en-3-one (IX)	0.38 (3)	Yellow
16α,17-Epoxy-20β-hydroxypregn-4-en-3-one	0.44 (3)	Dark brown
17α,20α,21-Trihydroxypregn-4-en-3-one (XI)	0.22 (4)	Yellow
17α,20β-21-Trihydroxypregn-4-en-3-one	0.25 (4)	Orange
16α,21-Dihydroxypregn-4-ene-3,20-dione (XIII)	0.22 (5)	Blue

The spots of the steroids on alumina were revealed in UV light and with iodine vapor; those on the fixed layer of silica gel by spraying the chromatogram with sulfuric acid with subsequent heating or by spraying with Lugol's solution; and those on paper by observation in UV light and by treatment with a solution of triphenyltetrazolium chloride.

Fermentation of 17α-hydroxyprogesterone (I). To each of four flasks containing a culture of A. roseochromogenus ATCC 3347 was added 25 mg of 17α-hydroxyprogesterone (I), and fermentation was carried out for 48 hr. After extraction and the distillation of the solvent, 113 mg of an oily residue was obtained which contained the initial substance I and the dihydroxyketone II. Thin-layer preparative chromatography on Al_2O_3 yielded 41.6 mg of the dihydroxyketone II

with mp 199–211° C. Two crystallizations from a mixture of acetone and methanol gave 16.2 mg of II with mp 209–211° C, $[\alpha]_D^{20}$ +77.5° (c 0.72), λ_{max} 241 mμ (log ε 4.18), IR spectrum: 3535, 3393, (OH), 1658, 1612 (Δ^4-3-CO) cm^{-1}. Found, %: C 76.14; H 9.86. Calculated for $C_{21}H_{32}O_3$, %: C 75.86; H 9.70.

The 20-acetate of 17α-dihydroxypregn-4-en-3-one (III). A solution of 48.2 mg of the dihydroxyketone II in 0.5 ml of absolute pyridine was treated with 0.5 ml of acetic anhydride. The mixture was left for 18 hr, and after the usual working up 48 mg of III with mp 200–201° C was obtained. After crystallization from ethyl acetate, substance III had mp 198.5–199.5° C, $[\alpha]_D^{20}$ +48.5° (c 0.83); IR spectrum: 3490 (OH), 1739 (acetate), 1663, 1613 (Δ^4-3-CO) cm^{-1}.

Found, %: C 74.03; H 9.42. Calculated for $C_{23}H_{34}O_4$, %: C 73.76; H 9.15.

Fermentation of 11α,17α-dihydroxyprogesterone (IV) with A. roseochromogenus ATCC 3347. A culture of A. roseochromogenus was fermented in ten flasks each containing 18.5 mg of IV for 3 days. After extraction and evaporation of the solvent, 180 mg of an oily residue was obtained. Preparative thin-layer chromatography on alumina [activity grade III; acetone–ethyl acetate (4 : 1)] yielded 83 mg of unpurified 11α,17α-trihydroxypregn-4-en-3-one (V). Two crystallization from a mixture of ethyl acetate and ethanol yielded 16.2 mg of pure V with mp 274–276° C. IR spectrum: 3460, 3385, 3327 (OH), 1643, 1613 (Δ^4-3-CO) cm^{-1}; mol. wt. 348 (mass spectrometry).

Fermentation of 16α,17-epoxyprogesterone (VI) with A. roseochromogenus ATCC 3347. To each of 20 flasks containing a culture of A. roseochromogenus was added 25 mg of VI, and fermentation was carried out for 56 hr. After extraction and working up, 440 mg of a yellow oil was obtained; this was dissolved in benzene and the solution was filtered through 5 g of alumina, the substance being eluted with benzene and then with ether. The clarified eluate was evaporated to dryness, giving 343 mg of a mixture of VI, VII, and X. Preparative thin-layer chromatography of this mixture twice on alumina [activity grade II; ether–benzene (3 : 2)] yielded 100 mg of VII and 22 mg of IX. After crystallization of VII from a mixture of acetone and petroleum ether, 52 mg of chromatographically pure 16α,17-epoxy-3β-hydroxy-5α-pregnan-20-one (VII) with mp 184–184.5° C was obtained. IR spectrum of VII: 3520, 3325 (OH), 1695 (20-CO) cm^{-1}.

The acetate of VII was obtained under conditions analogous to those for the acetate of III, mp 191–192° C (from acetone). IR spectrum: 1739 (acetate), 1703 (20-CO) cm^{-1}; NMR spectrum: 1.02 (18-CH_3), 0.83 (19-CH_3), 3.6 (epoxide proton), 4.65 (axial proton) ppm.

Found, %: C 73.75; H 9.00. Calculated for $C_{23}H_{34}O_4$, %: C 73.76; H 9.15.

After two crystallizations of XI from ethyl acetate, 2.9 mg of pure 16α,17-epoxy-20α-hydroxypregn-4-en-3-one (IX) with mp 235–240° C was obtained. IR spectrum: 3375 (OH), 1663, 1612 (Δ^4-3-CO) cm^{-1}.

Fermentation of cortexolone (X) with A. roseochromogenus ATCC 3347. A culture of A. roseochromogenus was fermented in 16 flasks each containing 15 mg of cortexolone (X). After fermentation for 4 days, extraction yielded 242 mg of an oily residue which, according to chromatographic data, consisted mainly of the starting material X with a very small amount of transformation product XI. Preparative thin-layer chromatography three times on Florisil yielded 5.6 mg of an oily substance consisting mainly of 17α,20α,21-trihydroxypregn-4-en-3-one. On value chromatography in the ether–benzene–methanol–water (7 : 3 : 5 : 5) system, the R_f paper of XI was 0.28, while that of the epimeric 17α,20β,21-trihydroxypregn-4-en-3-one is 0.32. When the chromatogram was treated with a solution of triphenyltetrazolium chloride, neither substance gave a red coloration [17]. When substance XI was chromatographed with authentic samples of 17α,20α,21-trihydroxypregn-4-en-3-one and the 20β isomer, it was found that substance XI was identical with the 17α,20α,21-trihydroxypregn-4-en-3-one (see table). IR spectrum: 3400 (OH), 1670, 1620 (Δ^4-3-CO) cm^{-1}.

Fermentation of 11-deoxycorticosterone (XII) with A. roseochromogenus ATCC 3347. A culture of A. roseochromogenus was fermented in eight flasks each containing 15 mg of 11-deoxycorticosterone. After fermentation for 3 days, extraction yielded 118.3 mg of an oily residue which, according to chromatographic data, contained mainly the starting material XII and a small amount of conversion product XIII. By preparative chromatography in a thin layer of alumina (activity grade V, ethyl acetate), twice, 4.3 mg of an oily product consisting mainly of 16α,21-dihydroxypregn-4-ene-3,20-dione (XIII) was isolated. When the substance obtained was chromatographed on paper together with an authentic sample of 16α,21-dihydroxypregn-4-ene-3,20-dione it was found that both substances had R_f 0.34 [in the petroleum ether–benzene–methanol–water (3 : 7 : 5 : 5) system] and when the chromatogram was treated with a solution of triphenyltetrazolium tetrachloride they both gave a red coloration.

Reduction of 17α-hydroxyprogesterone (I) with sodium borohydride. With stirring at 0° C, 65 mg of 87% sodium borohydride was added over 5 min to a solution of 330.5 mg of 17α-hydroxyprogesterone (I) in 70 ml of methanol. The mixture was stirred at the same temperature for 1 hr. After the usual working up and crystallization from a mixture of chloroform and ether and then from acetone–chloroform–petroleum ether, 17α,20β-dihydroxypregn-4-en-3-one was obtained with mp 202–202.5° C, $[\alpha]_D$ + 72° (c 1.0), λ_{max} 242 mμ (log ε 4.21); IR spectrum: 3440 (OH), 1650, 1617 (Δ^4-3-CO) cm^{-1}.

Fermentation of steroids with a cell-free preparation from A. roseochromogenus ATCC 3347. A flask containing 100 ml of nutrient medium of the composition given above was inoculated with 10 ml of the seed material grown as described above. The culture was grown under the same conditions for 6 hr and then 5 mg of 11α-hydroxyprogesterone in 0.5 ethanol was added to the flask and fermentation was continued for another 18 hr. After this time, the mycelium was filtered off and was carefully washed with 0.5% NaCl double-distilled water, and a 0.001 M solution of sodium ethylenediaminetetraacetate (EDTA). Then it was suspended in 10 ml of phosphate buffer (pH 7.4) containing 0.001 M EDTA and 0.001 M glutathione. The suspension was disintegrated in a ULA-250 ultrasonic generator at 0–6° C for 7–8 min. The disintegrated cells were centrifuged at 5° C for 35 min (1000 g) and the supernatant at the same temperature for 35 min (6750 g or 90 000 g). Two supernatants were accordingly obtained: at 6750 g and at 90 000 g.

A solution of 1 mg of a steroid in 0.1 ml of dimethylformamide and 5 mg of NADP-H_2 were added to 10 ml of the supernatant and incubation was carried out at 26° C for 14 hr, the reaction mixture being stirred on a vibro shaker. After the end of fermentation, the steroids were extracted with ethylene chloride and the extract was chromatographed in a thin layer of silica gel.

When 17α-hydroxyprogesterone (I) was fermented with the 6750 g and 90 000 g supernatants, a single product was obtained—17α,20α-dihydroxypregn-4-en-3-one (II). When 16α,17-epoxyprogesterone (IV) was fermented with the 6750 g supernatant, 16α,17-epoxy-3β-hydroxy-5α-pregnan-20-one (VII) and 16α,17-epoxy-20α-pregn-4-en-3-one (IX) were detected, and when 11α,17α-dihydroxyprogesterone (VI) was incubated with the 6750 g supernatant 11α,17α,20α-trihydroxypregn-4-en-3-one (V) was found. When cortexolone (X) and 11-deoxycorticosterone (XII) were fermented, no conversion products were found.

The culture of A. roseochromogenus ATCC 3347 was kindly given to us by Corresponding Member of the Academy of Sciences of the USSR G. K. Skryabin, the samples of 16α,17-epoxy-20α-hydroxypregn-4-en-3-one by L. V. Sokolova, the 16α,21-dihydroxyprogesterone by Prof. A. Wettstein (Switzerland), and the 17α,20α,21-trihydroxypregn-4-en-3-one by Dr. Garnik (Israel).

Conclusions

A culture of Actinomyces roseochromogenus ATCC 3347 and the corresponding cell-free preparation reduce 17α-oxygen-containing 20-oxopregnanes to the 20α-alcohol. A culture of A. roseochromogenus ATCC 3347 is capable of reducing Δ^4-3-oxo- to 3β-hydroxy-5α-steroids, as has been shown by fermentation with 16α,17-epoxyprogesterone as an example. It has been shown that certain substituents in the steroid molecule affect the course of the 20α-reduction by a culture of A. roseochromogenus ATCC 3347.

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