A FURTHER STUDY OF THE SUBSTRATE SPECIFICITY IN THE REDUCTION OF 20-OXOSTEROIDS WITH A CULTURE OF ACTINOMYCES ROSEOCHROMOGENUS

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As shown previously [1], a culture of Actinomyces roseochromogenus ATCC 3347 reduces the 20-oxo group of steroids of the pregnane series having an oxygen substituent in the 17α-position. In addition, this organism hydroxylates (in the 15α-position) 17-deoxypregnane steroids and some corticoids [2].

We have suggested that a 17α-oxygen function is necessary for the achievement of the reduction. This is connected with the possibility of the formation of an enzyme-substrate complex by means of which the conformation of the side chain of the steroid becomes favorable for the reduction of the 20-oxo group with the formation of a 20α-alcohol [3].

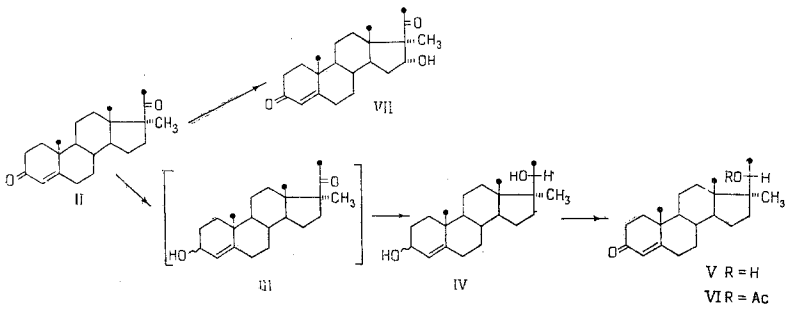
For a further elucidation of the role of the 17α-oxygen substituent in the fermentation of steroids with a culture of A. roseochromogenus it was necessary to establish whether this substituent could be replaced by others without this replacement affecting the process of reduction of the 20-oxo group and whether reduction could take place in the absence of a 17-oxygen substituent with a stable conformation of the side chain favorable for reduction. This paper gives the results of a consideration of these questions.

The fermentation of 17α-hydroxyprogesterone acetate (I) with a culture of A. roseochromogenus did not lead to a reduction of the 20-oxo group which, it would appear, indicates the necessity for the presence of a free hydroxyl group. But, here the factor of the steric hindrance of the carbonyl group possibly plays a part, since Wada Shun-yo [4] was unable to reduce the 20-carbonyl group in compound I with sodium borohydride.

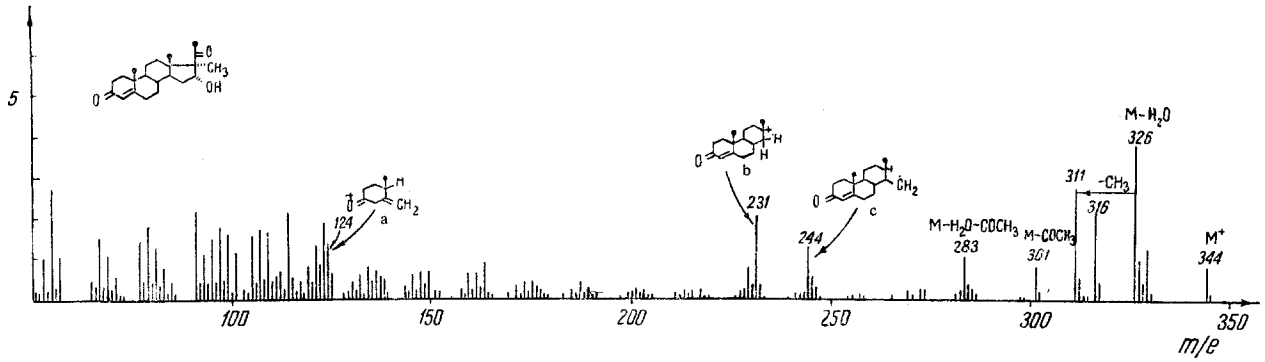
As a substrate having no oxygen at C-17 we took 17α-methylprogesterone (II), which we obtained by Deghenghi's method [5] from 3β-hydroxypregna-5,16-dien-20-one acetate. There is no information in the literature on the predominant conformation of the side chain in compound II. However, Kardis [6], on the basis of the chemical shift of the C-19 angular methyl group in the NMR spectrum of II, suggested that the conformation of its side chain differs from that of the side chain of progesterone (B_1*) [7]. Since it is known that a change in the conformation takes place more easily from B_1 to A_2 [7], it may be assumed that the side chain of 17α-methylprogesterone (II) predominantly possesses a conformation of type A.

In order to establish the predominant conformation of the side chain, 17α-methylprogesterone (II) was reduced with sodium borohydride. Reduction of both the 20-carbonyl and of the 3-oxo groups took place. In spite of the general rule [8] the conjugated 3-oxo group was reduced first, as was clearly seen from the formation of the intermediate III which did not absorb in UV light, and had R_f 0.46 (for II R_f 0.45, and for IV R_f 0.37). The oxidation of the tetrahydro derivative IV with manganese dioxide [9] led to the ketol V, the structure of which was shown by the IR and mass spectra of the ketol itself and of its acetate VI. The configuration of the hydroxyl group in V was established on t the basis of the increment in the molecular rotation on acetylation. Since the latter proved to be positive (ΔM_D = +145°), the 20-hydroxy group was ascribed the β-configuration [10] and compound V was ascribed the structure of 20β-hydroxy-17α-methylpregn-4-en-3-one. The formation of the 20β- alcohol on the reduction of 17α-methylprogesterone (II) indicates that its side chain exists in a conformation of the B type [7].

*According to Rakhit and Engel, in the reduction of 20-oxosteroids with complex metal hydrides the B_1 and B_2 conformations of the side chains favor the formation of the 20β-alcohol and the A_1 and A_2 conformations the formation of the 20α-alcohol.



In the fermentation of 17α-methylprogesterone (II) with a culture of A. roseochromogenus, a small yield of a substance VII, more polar than the starting material, was obtained. The molecular weight of VII was 16 units greater than II. This shows that VII contains an additional oxygen atom as compared with 17α-methylprogesterone (II). The mass spectrum of compound VII had peaks with m/e 301 and 283, which shows the splitting off of the side chain in the form of the unreduced methyl ketone. A peak with m/e 124 (fragment a, figure) is characteristic for a Δ^4-3-oxo grouping [11].



The position of the hydroxyl group was determined on the basis of the presence in the mass spectrum of peaks with m/e 231 and 244 (fragments b and c, figure), which excludes the possibility of the presence of the hydroxyl group in any position other than 16. The negative value of the increment in the molecular rotation $[\Delta M_D[(16\text{-OH}) - (16\text{-H})] = -111^\circ\}$ shows the α-configuration of the hydroxyl group at C-16 [12]. These results permit compound VII to be assigned the structure of 16-hydroxy-17α-methylpregn-4-ene-3,20-dione.

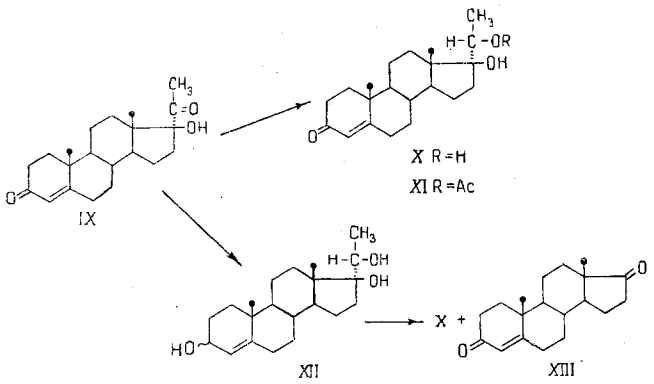
It must be noted that in the IR spectrum of compound VII the absorption bands corresponding to the 20- and the 3-oxo groups are not separated.

As a 17-deoxypregnene in which the side chain was arranged in a conformation favorable for the formation of the 20α-alcohol, we selected 16β-methylprogesterone (XVIII), which we obtained from 3β-hydroxypregna-5,16-dien-20-one by Wettstein's method [13]. The basis for this selection was the fact that, according to the results of NMR [14], optical rotatory dispersion [15], and conformational analysis [16], the side chain in 5β-methyl-substituted pregnanes has a conformation favorable to the formation of 20α-alcohols on reduction, i. e., a conformation of type A. This has been conformed experimentally by Klyne [17] by the reduction of 16β-methylpregnan-20-one with lithium aluminum hydride.

When 16β-methylprogesterone (VIII) was fermented with a culture of A. roseochromogenus, no formation of 20-dihydro derivatives of this substrate was observed. The substances formed in this fermentation in trace amounts possessed a considerably lower chromatographic mobility than the 16β-methylpregn-4-ene-3ξ,20ξ-diol obtained by the reduction of VIII with lithium aluminum hydride.

Until now, we have considered the action of a culture of A. roseochromogenus on 17β-pregnane derivatives. As a representative of the 17-iso derivatives we selected 17β-hydroxy-17α-progesterone (IX), which is formed by the hydration of ethynyltestosterone by Amiard's method [18]. This substrate was selected because it is an isomer of 17α-hydroxyprogesterone, the substrate which is most actively reduced by a culture of A. roseochromogenus. The fermentation of 17β-hydroxy-17α-progesterone (IX) with a culture of A. roseochromogenus gave substance X,

chromatographically less mobile than the initial ketone (IX). The IR spectrum of X contained absorption bands characteristic for a Δ^4-3-oxo grouping and hydroxyl groups, and the absorption band of a 20-oxo group was absent. The mass spectrum showed that X consists of a dihydro derivative of IX. From this it can be seen that compound X is nothing other than 17β,20-dihydroxy-17α-pregn-4-en-3-one.



The reduction of IX with sodium borohydride in methanol gave compound XII, the IR spectrum of which showed the absence of carbonyl groups and the presence of hydroxyl groups. The mass spectrum confirmed that XII is a tetrahydro derivative of IX. The oxidation of XII with magnesium dioxide gave a mixture of the diolone X and androst-4-ene-3,17-dione (XIII). The behavior of the androstenedione was not unexpected since the splitting out of the side chain under the action of manganese dioxide on 17,20-glycols in a number of derivatives of 17β-pregnane has been reported previously [19]. However, in the present case, i.e., in the case of the glycol of the 17-iso series, the formation of the 17-oxosteroids took place considerably more readily and with twice the yield.

The IR spectra, taken in the crystalline state, of samples of X isolated by chemical and by microbiological reduction differed in the position of the absorption bands of the main function groups and in their fingerprints. The samples also had different melting points. Nevertheless, the IR spectra of the samples in solution were identical, and so were their mass spectra. The two samples had the same chromatographic mobility, and mixtures of them gave no depression of the melting points. Thus, the chemical and microbiological reductions of 17β-hydroxy-17α-progesterone (IX) give the same product, namely the 20-dihydro derivative IX.

The configuration of the hydroxyl group at C-20 of X was suggested on the basis of the following considerations:
1. The features of the IR spectrum of X, taken in CCl_4, show the presence of an intramolecular hydrogen bond in the substance. From a consideration of the molecular models of the two epimeric 17β, 20-dihydroxy-17α-pregn-4-en-3-ones it can be seen that in the 20α-alcohol (S-configuration) a hydrogen bond can be formed between the hydroxy group at C-17 and C-20 without difficulty. In the 20β- alcohol (R-configuration) the formation of an intramolecular hydrogen bond would disturb the interaction between the C-21 methyl group and the axial hydrogen atoms at C-12 and C-14.

2. The reduction of 5α, 17α-pregnan-20-one with sodium borohydride in isopropanol gave the 20α-alcohol as the main product [20]. Since the reduction conditions are similar to those which we used and since under these conditions a 17-hydroxy group should have no influence on the stereodirectivity of the reduction of the 20-oxo group, it may be considered that in the case of the reduction of 17β-hydroxy-17α-progesterone (IX) we likewise obtained the 20α-(S-) alcohol.

A final decision on the configuration of the hydroxyl group was made after determining the absolute configuration of the asymmetric center at C-20 by Horeau's method [21], which is based on the esterification of secondary hydroxyl groups with the anhydride of racemic α-phenylbutyric acid. Under these conditions the (−)-R-acid reacts with the R-alcohol and the (+)-S-acid reacts with the S-alcohol, and from the sign of the rotation of the acid remaining in excess it is possible to deduce the absolute configuration of the alcohol reacting. The acid isolated in the esterification of X with the anhydride of racemic α-butyric acid had a negative rotation, which corresponds to the S-configuration of the alcohol. On the basis of what has been said above, compound X was ascribed the structure of 17β,20α-dihydroxypregn-4-en-3-one.

By combining Wettstein's results [13] and those of the present communication it may be stated that a predominant conformation of the side chain favorable for the formation of a 20α- alcohol is still not sufficient for the reduction of

the 20-oxopregnanes by a culture of A. roseochromogenus. This was shown in the case of fermentation with 16β-methylprogesterone (VIII). A necessary condition for the performance of the reduction is the presence in position 17 of the steroid molecule of a substituent which is, possibly, responsible for the formation of the enzyme-substrate complex. Just such a substituent will be a free hydroxy group or an epoxy group. In substrates with a conformation of the side chain unfavorable for the formation of a 20α-alcohol but having a 17-oxygen function, reduction does take place and during it the conformation of the side chain apparently changes into a conformation favorable for the formation of the 20α-alcohol.

The inversion of the configuration of the hydroxyl group and of the side chain at C-17 prevents the reduction of the 20-oxo group.

EXPERIMENTAL

Before analysis, the samples were dried at 100° C in vacuum (1 mm Hg) for 6 hr. If the conditions of exposure are not described specifically, the IR spectra were recorded on a UR-10 spectrophotometer (mull with paraffin oil). The specific rotations were determined in chloroform at 20° C. The mass spectra were obtained on a MKh-1303 instrument. The molecular weights (mol wt) were determined by mass spectrometry.

Chromatography. Plates with a fixed layer of type KSK silica gel were prepared by a published method [22]. The spots of the steroids on the alumina were revealed by UV light and iodine vapor, and those on a fixed layer of silica gel by spraying the chromatograms with H_2SO_4 followed by heating them or by spraying with Lugol's solution.

Cultivation of the seed material. 100 ml of 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 liter of tap-water (pH 7.0) were inoculated with the aerial mycelium of Actinomyces roseochromogenus from an agar slope, and the organism was cultivated under aerobic conditions on a rotary shaking machine (200 rpm) at 28° C for 72 hr.

Fermentation of Actinomyces roseochromogenus ATCC 3347 with steroids. Flasks each containing 100 ml of nutrient medium of the composition described were inoculated with the seed material obtained (10 ml each), and the cultures were grown for a day under the conditions described. Then a solution of a steroid in 0.5 ml of ethanol was added to each flask. After the end of the fermentation, the mycelium was filtered off and washed with hot water, and the filtrate and the wash-waters were combined and extracted with methylene chloride. The extract was dried over magnesium sulfate and evaporated to dryness. The fermentation products were extracted from the residue, or their composition was determined chromatographically.

Fermentation of 17α-hydroxyprogesterone acetate (I). A culture of A. roseochromogenus was fermented with 14 mg of I for 72 hr, 5-ml samples being taken after every 24 hr. After the usual treatment of the samples, the residue obtained was chromatographed on silica gel (ether). The chromatography of samples taken during the 3 days of the fermentation showed that only the initial substance I was present in the culture liquid.

Reduction of 17α-methylprogesterone (II) with sodium borohydride. With stirring, 15 mg of 87% sodium borohydride was added to a solution of 70 mg of II (mp 136–139° C, $[\alpha]_D$ +110°) in 18 ml of methanol. The reaction mixture was stirred with cooling for another 4.5 hr and then at room temperature for another 2.5 hr. After each hour a further 10 mg of sodium borohydride was added to the reaction mixture. At the end of the reaction, a few drops of glacial acetic acid were added and then the methanol was evaporated off until a precipitate began to form, and 30 ml of water was added. The precipitate was extracted with chloroform (3 × 30 ml), and the extract was washed with 5% sodium bicarbonate solution and then with water to neutrality, and was dried over magnesium sulfate for 18 hr. It was found by thin-layer chromatography [silica gel, benzene–acetone (6 : 1)] that during its standing in chloroform solution the product of the reduction of IV (R_f 0.37) was converted to a considerable extent into a less polar substance (R_f 0.68). Evaporation of the chloroform extract to dryness yielded 71 mg of a colorless oil. Thin-layer preparative chromatography on alumina (neutral, activity grade IB, ether) gave 35 mg of the slightly contaminated diol IV, which was rechromatographed under the same conditions, giving 22.8 mg of chromatographically homogeneous 17α-methylpregn-4-ene-3,20-diol (IV).

Preparation of 20β-hydroxy-17α-methylpregn-4-en-3-one (V). A solution of 22.8 mg of the diol IV in 7 ml of absolute benzene was stirred at room temperature while 200 mg of activated manganese dioxide [9] was added. The mixture was stirred for another 2 hr, the manganese dioxide was filtered off and washed on the filter with benzene, and the benzene solutions were evaporated to dryness in vacuum. This gave 20.3 mg of an oily product which

crystallized on being treated with ether. Thin-layer chromatography on alumina (neutral, activity grade III, ethyl acetate) twice finally yielded 11.6 mg of substance V with mp 144–147° C. An analytical sample prepared by crystallization from ether had mp 147–148.5° C, $[\alpha]_D$ +56.3° (c 0.72); IR spectrum, cm^{-1}: 3495 (OH), 1615 and 1666 (Δ^4-3-CO); mol wt 330.

Preparation of 20β-hydroxy-17α-methylpregn-4-en-3-one acetate (VI). A solution of 5 mg of the ketol V in a mixture of 0.1 ml of absolute pyridine and 0.05 ml of acetic anhydride was left at room temperature for 18 hr and was then evaporated in vacuum. This gave 6.5 mg of VI with mp 164–166° C; $[\alpha]_D$ +88.8° (c 1.08); IR spectrum, cm^{-1}: 1725 (CO of an acetate), 1620 and 1677 (Δ^4-3-CO); mol wt 372.

Fermentation of 17α-methylprogesterone (II). To each of 15 flasks of a stock culture of A. roseochromogenus was added 10 mg of II and fermentation was carried out for 72 hr. After extraction (4 × 400 ml) and distillation of the solvent, 220 mg of a yellow oil was left; this was treated with ether and the insoluble matter was filtered off. This was an amorphous substance which, according to thin-layer chromatography on alumina (activity grade II, ethyl acetate) contained mainly the initial ketone II (R_f 0.84) with a small amount of substance VII (R_f 0.46). By thin-layer preparative chromatography on alumina (activity grade II, ethyl acetate), 10 mg of VII was isolated which, after crystallization from ethyl acetate, had mp 194–197° C, $[\alpha]_D$ +72.7° (c 0.52); IR spectrum, cm^{-1}: 3457 (OH), 1616 and 1677 (broad band) (Δ^4-3-CO, 20-CO); mol wt 344.

Reduction of 16β-methylprogesterone (VIII) with lithium aluminum hydride. A solution of 2 mg of VIII (mp 207–209° C, $[\alpha]_D$ +132.6°) in 1 ml of a mixture of absolute benzene and ether (1 : 1) was treated with 3 mg of lithium aluminum hydride and the reaction mixture was boiled for 3 hr, after which a few drops of water was added, and the organic layer was separated off and evaporated to dryness. Chromatography of the residue on silica gel (benzene–ether (1 : 1)) showed the presence of the initial compound (VIII) and of two more polar substances (XIV and XV) (table).

Results of a Chromatographic Comparison of the Substances Obtained by the Reduction and by the Fermentation of 16β-Methylprogesterone (VIII)

Treatment	Reaction product	R_f	Absorption in UV light	Color reaction	
				INAH*	Lugol's solution
Reduction with $LiAlH_4$	XIV	0.35	—	—	Blue
	XV	0.25	—	—	White
Fermentation with A. roseochromogenus	XVI	0.20	—	—	Blue
	XVII	0.13	+	+	

*Isonicotinic acid hydrazide.

Fermentation of 16β-methylprogesterone (VIII). To a flask containing a stock culture of A. roseochromogenus was added 10 mg of VIII, and fermentation was carried out for 72 h. After extraction (3 × 40 ml) and evaporation of the solvent, an oily residue was obtained, the chromatography of which in a thin layer of silica gel [benzene–ether (1 : 1)] showed the presence of the initial substance VIII (R_f 0.55) and trace amounts of two more polar fermentation products (XVI and XVII, see table).

Fermentation of 17β-hydroxy-17α-progesterone (IX). To each of 20 flasks containing the stock culture of A. roseochromogenus was added 10 mg of IX (mp 193–196° C, $[\alpha]_D$ +60°), and fermentation was carried out for 72 hr. After extraction (4 × 400 ml) and evaporation of the solvent, 230 ml of a yellow oil was obtained which crystallized on trituration with ether. The precipitate was filtered off and washed with ether, giving 150 mg of a substance consisting, according to thin-layer chromatography (silica gel, ether) of the starting material IX and the fermentation product X. After two crystallizations from ethyl acetate, 70 mg of 17β,20α-dihydroxy-17α-pregn-4-en-3-one (X) with mp 165–167° C was obtained. An analytical sample of X obtained by recrystallization from ethyl acetate containing a small amount of methanol had mp 169.5–171.5° C; $[\alpha]_D$ +70.4° (c 0.667); IR spectrum, cm^{-1} 3485, 3448 (OH), 1624, 1643, and 1677 (Δ^4-3-CO); IR spectrum (c $2.83 \cdot 10^{-3}$M), cm^{-1}: 3636, 3565 (OH, intramolecular hydrogen bond), 1622 and 1680; mol wt 332.

Acetylation of 17β,20α-dihydroxy-17α-pregn-4-en-3-one (X). The milligrams of the diol X was acetylated under the conditions described for VI, and the product was crystallized from ethyl acetate, giving 7.6 mg of the 20-acetate of 17β,20α,17α-pregn-4-en-3-one (XI) with mp 207–210° C, $[\alpha]_D$ +52° (c 0.40); IR spectrum (in a tablet of KBr), cm^{-1}: 3584, (OH), 1728 (CO of an acetate), 1614 and 1666 (Δ^4-3-CO); mol wt 374.

Reduction of 17β-hydroxy-17α-progesterone (IX) with sodium borohydride. With stirring and cooling to −20° C, 212 mg of 87% sodium borohydride were added to a solution of 300 mg of IX in 35 ml of methanol. Then the temperature was raised to −10° C, and the reaction mixture was carefully neutralized with 50% acetic acid to pH 6.5, evaporated in vacuum to small volume, diluted with 100 ml of water, and extracted with ether (4 × 50 ml). The extract yielded 284 mg of the triol XII. IR spectrum, cm^{-1}: 3520 and 3380 (OH); mol wt 334.

Preparation of 17β,20α-dihydroxy-17α-pregn-4-en-3-one (X). A solution of 280 mg of the triol XII in a mixture of 20 ml of absolute benzene and 10 ml of acetone was stirred with 1.3 g of activated manganese dioxide for 6.5 hr [8]. The usual working up and preparative chromatography on alumina (activity grade V, ether) provided 111 mg of the diol X and 74.1 mg of androst-4-ene-3,17-dione (XIII). Recrystallization of the dione XIII from methanol gave androst-4-ene-3,17-dione (XIII) with mp 171.5–172.5° C; IR spectrum, cm^{-1}: 1740 (17-CO), 1618, and 1668 (Δ^4-3-CO). A mixture with authentic androstenedione gave no depression of the melting point. An analytical sample of the diolone X obtained by repeated recrystallization from ethyl acetate–methanol had mp 174–177° C. A mixture with a sample of X from the microbiological experiment gave no depression of the melting point.

The chromatographic mobilities on silica gel of the samples of X obtained by chemical and microbiological methods were identical: R_f 0.015 (chloroform); 0.048 [hexane–ethyl acetate (3 : 1)]; 0.21 [ether–chloroform (2 : 1)]; 0.3 [benzene–ethanol (9 : 1)]; 0.35 (ether); 0.45 (ethyl acetate), and 0.71 [chloroform–ethanol (4 : 1)]. IR spectrum, cm^{-1}, 3537, 3387 (OH), 1619 and 1660 (Δ^4-3-CO); IR spectrum (CCl_4), c 2.7×10^{-3}M, cm^{-1}: 3637, 3565, (OH, intramolecular hydrogen bond), 1622 and 1680 (Δ^4-3-CO); mol wt 332.

Determination of absolute configuration by Horeau's method. To 4.8 mg of the steroid X was added 0.5 ml of a solution of the anhydride of racemic α-phenylbutyric acid in freshly-distilled pyridine (217 mg of the anhydride was diluted with pyridine to 9.2 ml) containing 11.8 mg of the anhydride. The mixture was left at room temperature for 16–17 hr. Then 1 drop of water was added and the reaction mixture was heated at 80–90° C for 30 min. After it had been cooled to room temperature, 3 ml of benzene and 2 ml of water were added and it was titrated with 0.1 N NaOH solution in the presence of phenolphthalein. The neutralized mixture was treated with 10 ml of benzene, the organic layer was separated off, the aqueous layer was extracted with chloroform (2 × 2 ml), the extract was discarded, and the aqueous layer was acidified with 1 ml of 1 N HCl and extracted with benzene (3 × 3 ml). The benzene extract was evaporated to dryness, the residue was dissolved in 0.6 ml of benzene, and the angle of rotation was determined with a polarimetric tube having a volume of 0.5 ml and a length of 0.5 dm. The acid obtained had $\alpha = -0.069 \pm 0.012°$. On the assumption of a quantitative esterification of the alcohol with one anantiomer and the absence of losses during isolation, the acid obtained should have $\alpha = -0.193$. This shows the 20α-(S-) configuration in compound X.

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CONCLUSIONS

A culture of Actinomyces roseochromogenus ATCC 3347 reduces 17-hydroxy- and 16α,17α-epoxy-20-oxopregnenes to the corresponding 20α-alcohols, but is incapable of reducing 17α-acetoxy-, 17α-methyl-, and 16β-methylprogesterones or 17-unsubstituted 20-oxopregnenes. The results obtained show that the presence of the side chain in a conformation favorable for the formation of 20α-alcohols in the reduction of 20-oxosteroids with complex metal hydrides is in itself insufficient for the reduction of the 20-oxosteroids by a culture of Actinomyces roseochromogenus, which also reduces the 20-oxo group of 17α-pregnenes.

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