STEROID TRANSFORMATIONS.

187. MICROBIAL CONVERSION OF 3β-HYDROXY-5α-H-PREGNANES TO THEIR Δ^4-3-KETO-9α-HYDROXY DERIVATIVES

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The problem of synthesizing corticosteroids from 5α-H-Δ^{16}-pregnenolone, obtained from tigogenin and neotigogenin, is closely tied to the development of effective methods to functionally deactivate rings A, B, and C. In recent years, this has been achieved by a combination of multistage chemical and microbiologic methods, by means of which is carried out the sequential functionalization of ring A, and then ring C, D, or vice versa [7, 8, 10, 15, 16, 17, 18]. In the present work we investigated a novel microbiological approach to this problem: we studied the possibility of simultaneous Δ^4-dehydrogenation and 9α-hydroxylation of 3β-hydroxy(acetoxy)-5α-H-pregnanes for their conversion to 9α-hydroxy-Δ^4-3-ketosteroids, which are key intermediates in the synthesis of 9α-halogeno-11β-hydroxysteroids, used in the pharmaceutical industry [13]. For this purpose, we used as catalyst a microorganism belonging to the genus Rhodococcus, in which we previously found Δ^4-3-ketodehydrogenase and 9α-hydroxylating activity towards 3β-hydroxy(acetoxy)-Δ^5-steroids [5, 11].

EXPERIMENTAL (MICROBIOLOGICAL)

For the transformations we selected a strain of Rhodococcus, previously isolated on mineral medium with dodecane as sole carbon source, from soil contaminated with petroleum products [11]. Cultures of the transforming microorganism were maintained and grown similarly to [2, 11]. The inoculate for transformation (24-hour culture) was added in the amount of 5-10 vol. % (0.03-0.06 g dry biomass) to medium of the following composition (g/liter): maize extract (8.0), K_2HPO_4 (1.0), $NaH_2PO_4 \cdot 12H_2O$ (4.5), and tap water, pH prior to sterilization 7.5-8.0. Culturing and transformation were carried out at a temperature of 24-30°C, on a shaker with mixing at 200-220 rpm. To a 5-6 h culture was added steroid in dimethyl sulfoxide (DMSO), the amount of which in the culture liquid did not exceed 2%, and the concentration of steroid in which, depending on the structure, was 0.5-5-2 g/liter. When necessary to increase the steroid concentration, it was added as a microcrystalline powder with a particle size of 1-10 μ. Steroids were isolated from the culture liquid by extraction with ethyl acetate, with subsequent removal of the solvent under vacuum.

EXPERIMENTAL (CHEMICAL)

To analyze the transformation products, we used analytical and preparative thin layer chromatography on plates of Silufol UV-254 and silica gel (Silpearl), as well as column chromatography on SiO_2 (Lakhhema, 40/100 μ). Yield of transformation products is given in percent, from the amount of isolated, chromatographically pure product. Melting points were recorded on a Kofler block. PMR spectra were determined on a Bruker wm-250 instrument, relative to TMS. Structures of compounds synthesized were demonstrated by identification with known samples, and by physicochemical data.

16α,17α-Epoxypregn-4-ene-9α,20-diol-3-one (VIIIe). The reaction mixture, produced by transformation of 0.13 g IIb (see Table 1; 3 g/liter), was separated by TLC (petroleum ether–Me_2CO, 3:1). Obtained was a mixture of 0.06 g IIe and 0.03 g VIIIe, mp 198-200°C (from Me_2CO). IR spectrum (ν, cm^{-1}): 1620, 1660, 3380. PMR spectrum (δ, ppm): 0.94 s, (18-Me), 1.32 s (19-Me), 1.18 d (21-Me, J = 7 Hz), 2.4 s (9-OH), 3.31 br s (20-OH), 3.47 c (16-H), 4.38 d (20-H, AB spectrum J = 7 Hz), 5.86 br s (4-H). Mass spectrum (m/z): 346 $[M - H_2O]^+$.

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TABLE 1. Results of Transformation of Compounds I-VI by Cultured Cells of Rhodococcus sp.

Initial compound	Steroid concentration, g/liter	Inhibitor, g/liter	Transform. temp.	Transform. time, h	Obtained compound, yield, %						Steroid yield total, %
					9α-oxo-Δ⁴-3-keto	Δ⁴-3-keto	5α-H-Δ⁴-3-keto	9α, 20-diol Δ⁴-3-keto	other compounds	Initial substrate	
Ib	1,0	—	28	16	—	—	—	—	VIIe,70	—	80
Ib	2,0	—	28	18	Ie, 20	+	—	+	VIIe,33	—	95
Ib	3,0[a]	α, α-Dipyridyl 0,1	28	42	—	Id,65	—	—	—	+	75
Ib	4,0[a]	Menadiol, 0,04	28	21	Ie, 33	Id,28	Ic, 5	—	VIIe,10	+	90
IIb	1,0	—	28	26	IIe 28	—	—	VIIIe,28[b c]	—	—	60
IIb	1,0	α, α-Dipyridyl 0,1	28	40	—	IId,40	—	—	VIIId 22[c]	—	65
IIb	3,0[a]	—	28	20	IIe, 45	—	—	VIIIe,22	—	—	70
IIIb	1,0	—	28	20	IIIe, 6	—	—	IXe,40[c]	—	—	50
IIIb	1,0	—	26	24	IIIe, 26	—	—	IXe,14	—	+	55
IIIb	3,0[a]	—	28	12	IIIe, 27	—	—	IXe,5	—	18	50
IVb	0,5	—	28	24	IVe, 50	IVd, 8	—	—	—	14	75
IVb	0,5	—	26	70	IVe, 40	IVd, 18	—	—	—	—	60
IVb	1,0	—	26	70	IVe, 45	IVd, 4	—	—	—	15	80
IVb	1,0	—	28	24	IVe, 30	+	—	—	+	+	70
IVb	1,0[a]	—	28	26	IVe, 75	—	—	—	+	6	90
IVb	0,5	$CoCl_3$, 0,05	28	12	IVe, 20	IVd, 35	—	—	—	25	80
IVb	0,5	$CoCl_3$, 0,1	28	12	IVe,10	IVd, 25	—	—	—	40	80
IVd	1,0	—	28	48	IVe,62	+	—	—	—	15	80
IVd	2,0	—	28	48	IVe,19	+	—	—	—	40	60
Vb	0,5[a]	—	28	24	Ve,50	Vd 10	—	—	—	—	65
Vb	1,0	—	28	40	Ve,22	Vd 18	—	—	+	—	35
Vb	1,0	—	28	26	Ve,7	Vd 29	—	—	+	7	70
Vd	0,8	—	28	48	Ve,5	Vd, 50	—	—	+	+	75
VIb	0,5	—	26	20	—	—	VIc, 45	—	35	+	90
VIb	1,0[a]	—	30	27	—	—	VIc 12	—	+	45	90
VIb	1,0[a]	—	30	40	—	—	VIc 10	—	+	30	65

[a]Steroid pulverized by ultrasound and added to culture without solvent.
[b]In [5], this compound was erroneously asigned structure VIIId.
[c]Compounds VIII and IX should probably be assigned a structure with 20β-orientation, in accordance with the known ability of Arthrobacter and Rhodococcus sp. to effect 20-β-reduction of 20-ketosteroids [18].

16α, 17-Epoxypregn-4-ene-9α-ol-3,20-dione (IIe) from 16α,17α-epoxypregn-4-ene-9α,20-diol-3-one (VIIIe). To a solution of 1.4 g $(CrO_3)_2$ in 7 ml DMFA cooled to 0° was added, with stirring, 0.22 g of the mixture obtained by transformation of 0.25 g of IIb (see Table 1). This was incubated 6 h, then poured into water and extracted with $CHCl_3$. The extract was dried over $MgSO_4$, the solvent removed under vacuum, and the dry residue fractionated on a column of Al_2O_3, eluting with ether. Yield was 0.15 g IIe, mp 229-230° [2].

Transformation of Ib in the Presence of Menadione. A solution of 0.2 g Ib in 1.5 ml DMFA was incubated for 14 h with Rhodococcus sp., following which 0.4% menadione in 0.4 ml ethanol was added and incubation continued for 7 h. The extract obtained was dried over $MgSO_4$, and after removal of the solvent the residue was fractionated by TLC (petroleum ether–Me_2CO, 2.5:1). Obtained was 0.07 g Ie, mp 190-193°C (Me_2CO/C_6H_{14}) [11], 0.06 g Id, 0.02 g VIIe, mp 206-210°C (MeOH/ether) [19], and 0.01 g 5α-H-pregn-16-ene-3,20-dione (Ic), mp 214-217°C (Me_2CO/C_6H_{14}) [20]; IR spectrum (ν, cm^{-1}): 1580, 1660, 1720; PMR spectrum (δ, ppm): 0.92 s (18-Me), 1.05 s (19-Me), 2.27 s (21-Me), 6.7 br s (16-H).

RESULTS AND DISCUSSION

For studying the Δ^4-dehydrogenase and 9α-hydroxylase activities of the Rhodococcus sp., we chose the readily available and potentially usefull 5α-pregnanes Ib-VIb, for which synthetic pathways have previously been developed from tigogenin [3, 6, 15, 16]. The results obtained (see Table 1) indicate, firstly, that in principle it is possible to convert 3β-hydroxy-5α-H-pregnanes to their Δ^4-3-keto-9α-hydroxy derivatives, and secondly, that it is possible to separate the Δ^4-dehydrogenation step from the subsequent 9α-hydroxylation by the addition of 9α-hydroxylase inhibitors (α,α-dipyridyl or $CoCl_3$). It should be noted that the course of the transformation and the yields of the desired products depend very much on the structure of the substrate, its method of addition and concentration, the incubation temperature, aeration intensity, and other factors. For compounds I, II, and III, one factor which interferes with the desired transformation is the exposure of the 20-keto group to the activity of 20-hydroxysteroid dehydrogenase, which is produced by the transforming microorganisms. Reduction of the 20-keto group of Ia,b [16], even with short incubation times, ends with the removal of the side chain and formation of 9α-hydroxyandrostenedione VIIe. Previously, in the case of Δ^5-steroids, we showed that reduction of the carbonyl group at C(20) can be prevented by carrying out the transformation in the presence of menadione [12]. However, in the case of Ib, addition of menadione simultaneously with the steroid inhibits oxidation of the A ring. Transformation of Ib at a concentration of 4 g/liter and with addition of menadione after 14 h results in a mixture consisting of Ic, Id, Ie, and VIIe.

Conversion of IIb [15] and IIIb [16] at identical concentrations of 3 g/liter concludes with the formation of 9α-hydroxypregnanes IIe and IIIe [2] and their 20-hydroxy derivatives VIIIe and IXe respectively, with yields of 45, 27, 22, and 5%. The undesired reduction at C(20) is intensified with increased biomass of the inoculate and higher incubation temperatures, which is well illustrated by the transformation of IIIb (see Fig. 1). Addition of IIIb as a powder with particle size 1-10 μ allows not only increasing the concentration to 3 g/liter, but also inhibits C(20) reduction, as a result of which IXe is formed in the amount of 5%, compared with 40% when IIIb is added in DMFA. However, in this case epoxide IIIb with the corticoid side chain is transformed with a high degree of damage; the total yield of steroids does not exceed 50%, and the yield of the desired product IIIe with this method is low – 26-27% (see Table 1). By using the additional chemical methods described below it was possible to carry out preparative synthesis of 9α-hydroxyepoxides IIe, IIIe. Thus, increase in the yield of IIe to 70% was achieved prior to oxidation of the total mixture of 9α-hydroxyepoxides IIe and VIIIe, obtained on transformation of IIb, using $(CrO_3)_2Py$ in DMFA. It was also found that 20,20-dimethoxyepoxide IVb, the one-step synthesis of which from IIa has been described by us previously [6], when added at 1 g/liter as a finely dispersed powder to the culture liquid at pH 8.0, is transformed by Rhodococcus sp. to IVe with a yield of 75%. The latter is hydrolyzed by a recently published method to IIIe [2]. It should be noted that here, the method of adding the substrate plays an important role. Thus, the yield of IVe is lowered to 50 and 30% when IVb is added in DMFA solution at concentrations of 0.5 and 1.0 g/liter, respectively (see Table 1). The decrease in yield of IVe, with increase in concentration of IVb, dissolved in DMFA, is due to the accumulation of Δ^4-3-ketoepoxide IVd [2], which inhibits further 9α-hydroxylation. This was shown by a transformation in which the starting substrate is Δ^4-3-ketone IVd. While the conversion of IVb at a concentration of 1 g/liter is complete after 24 hours, for the analogous transformation on

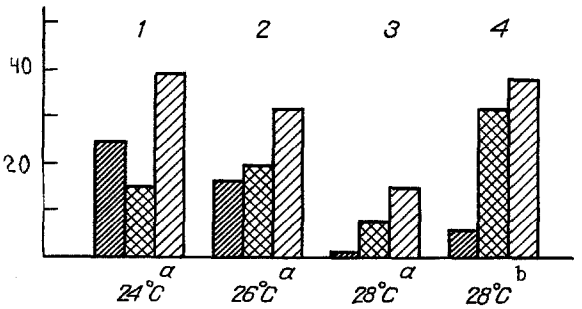
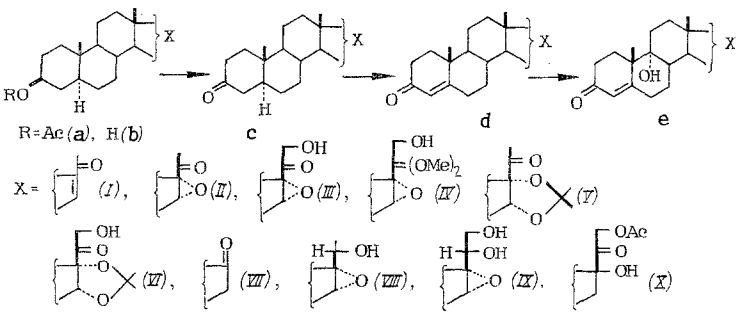


Fig. 1. Yield of transformation products of 16α-17α-epoxy-5α-H-pregnane-3β,21-diol-20-one as a function of incubation temperature and amount of inoculate of Rhodococcus sp. 1) 24°C; 2) 26C; 3,4) 28°C. Ordinate–steroid content: 9α-hydroxy-Δ^4-3-ketone IIIe (dense hatchmarks); 9α, 20-dihydroxy-Δ^4-3-ketone IVe (crossed hatchmarks); total amount of steroids extracted from culture liquid (light hatchmarks). Amount of inoculate–10% (1-3), 5% (4).

IVd, there is starting material still present in the culture liquid even after 48 h. Increasing the concentration of IVd to 2 g/liter and decresing the incubation temperature inhibits 9α-hydroxylation. Thus, at 26°C, even after 70 h of incubation of IVb at 1 g/liter, there remains untransformed starting material and its intermediate, IVd (see Table 1).



A similar inhibition of 9α-hydroxylation, due to accumulation of the Δ^4-3-keto derivative, is clearly seen in the experiment with acetonide Vb [3], which on incubation at a concentration of 1 g/liter for 26 h is converted to a mixture of Ve [5] and Vd [1], the composition of which changes little on prolonging the transformation to 48 h. Increasing the transformation time of Vb, added at a concentration of 1 g/liter, leads only to the development of destructive processes and a sharp decrease in the total yield of steroid products. When Vd is used as the starting material, the above is true for IVd (see Table 1). This result contradicts that of [11] for the analogous conversion of Vd to Ve in high yield (70%), which was incorrectly calculated, based on Vd transformed with low conversion of the initial substrate.

In contrast to the preceding steroids, the transformation of VIb is inhibited at the stage of formation of the 5α-H-3,20-diketone (VIc) [4] with concentrations of 0.5 and 1.0 g/liter. Even after incubating VIb 40 h, the culture liquid contains only VIc and starting VIb. This unexpected inhibition at the stage of Δ^4-dehydrogenation undoubtedly requires further study.

Accumulation of Δ^4-ketosteroids, which takes place in a number of cases of 9α-hydroxylation of 5α-H-ketosteroids and at first glance is undesirable, may nevertheless be useful, with the need to separate the Δ^4-3-ketodehydrogenation and 9α-hydroxylation steps in those cases where the ultimate purpose of the experiment is to produce 9α-deoxy-Δ^4-3-keto derivatives from 5α-H-steroids. The ability of the Rhodococcus sp. to convert 5α-H-steroids to Δ^4-3-keto derivatives is by itself of practical interest, since information on microorganisms which carry out this conversion is very limited [17, 18]. The possibility of separating the Δ^4-3-ketodehydrogenation and 9α-hydroxylation steps, carried out on 5α-H-steroids by one culture, has not been previously studied. In connection with this we studied the transforma-

tion of 5α-H-epoxides Ib, IIb, and IVb, as well as compound Xa, which is the 5α-H-analog of substance R acetate – a useful and important intermediate in the synthesis of corticosterone. On transformation of Δ^5-3β-hydroxysteroids by the Rhodococcus sp., it has been shown that incubating the steroids in the presence of 9α-hydroxylase inhibitors, and in some cases in their absence, led to the accumulation of only the Δ^4-3-keto derivatives [5]. The analogous transformation of Ib in the presence of 0.1% α,α-dipyridyl is complete after 42 h, with a 65% yield of Δ^4-3-keto compound Id. Transformation of epoxide IIb proceeds with the formation of 40% IId and 22% of its 20-hydroxy analog VIIId [9]; it should be noted that α,α-dipyridiyl weakly inhibits the rate of reaction. Transformation of Ib and IIb at respective concentrations of 3.0 and 1.0 g/liter continues in its presence for about 40 h, whereas without the inhibitor, transformation of Ib and IIb at concentrations of 4.0 and 3.0 g/liter is complete at 20 h. In contrast to I and II, incomplete inhibition of 9α-hydroxylase is seen in the case of IVb with the use of $CoCl_3$. On the other hand, transformation of Xa at a concentration of 1 g/liter by the Rhodococcus sp. does not require the use of 9α-hydroxylase inhibitors, and gives a final yield of corticosterone Xd of 55%.

Quantitative and qualitative chromatographic monitoring of the reaction course of Ia showed that the Rhodococcus sp. catalyzes sequential reactions: hydrolysis of the 3β-acetate group; oxidation of the 3β-hydroxyl group, with formation of 5α-H-3,20-diketone Ic; Δ^4-dehydrogenation; 9α-hydroxylation of the Δ^4-ketone Id; and finally, degradation of the side chain, resulting in the formation of the 17-ketones VIIe and VIId, as well as a decrease in the total yield of steroids (see scheme). As can be seen from the above data, the stage at which transformation of the studied compounds Ib-VIb and Xa stops depends mainly on the functionalization of ring D (structure of the substituents on ring D). As a result of this, of the seven 5α-steroids studied under the present conditions, it was not possible to obtain the 9α-hydroxy derivatives of VIb and Xa.

In [11], from among the Δ^5-3β-hydroxysteroids which are transformed by the Rhodococcus sp., the Δ^5 analogs of I, II, V, and X were studied. Comparative orientational data allow us to conclude that the transforming activity of the Rhodococcus sp. does not vary significantly from Δ^5-3β-hydroxysteroids to the corresponding 5α-H compounds.

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