Chemistry of Natural Compounds and Bioorganic Chemistry

Transformed steroids
195. Introduction of the 9α-hydroxygroup into Δ^5-3β-hydroxysteroids by *Circinella sp.* mold

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A preparative method for 9α-hydroxylation of Δ^5-3β-hydroxysteroids using the fungi of *Circinella sp. IOKh-1220* not capable of modifying the **A** ring has been developed. It is established that the yields of the main and the side products greatly depend on the transformation conditions, mycelium age, and the structure of the steroid substrate. Under the optimal transformation conditions novel 9α-hydroxysubstituted derivatives of androstenolone, pregnenolone, 16-dehydro-16α,17α-epoxy-, and-16α-methoxypregnenolone have been obtained in 36—80 % yields.

Key words: hydroxylation, Δ^5-3β-hydroxysteroids, Δ^5-3β,9α-dihydroxysteroids, *Circinella sp.*

The functionalization of ring **C** of a steroid molecule is the key problem in the synthesis of corticoid drugs. At present it is solved by two methods, *i.e.*, by photochemical 9-chlorination using a substituent at the O-atom of the 3-hydroxy group as a carrier, or via microbial 11- or 9-hydroxylation either by fungi or by bacteria. The first method requires the preliminary synthesis of 3α-axial *m*-iodobenzoates, which, in turn, reqiures the inversion of the 3β-hydroxy group configuration, and so it is applicable only to saturated 5α(H)-steroids.[1] The second method, as a rule, is suitable only for compounds of the Δ^4-3-oxo series, which also severely restricts its applicability.[2]

In the course of a study of the molds[3] useful for steroid transformation we discovered the *Circinella sp. IOKh-1220* (*C. sp.*) strain, which has the rare capacity to 9α-hydroxylate Δ^5- and 5α(H)-3β-hydroxysteroids, thus extending considerably the means of functionalizing the **C** ring.

The purpose of this work was to study the process of pregnenolone (**1**) transformation by *Circinella sp. IOKh-1220*, to choose the optimal method providing the maximal yield of 9α-hydroxy pregnenolone (**1a**), and to establish the dependance of this process on the nature of the substituents in ring **D** for the steroids of the pregnane and androstane series.

It has been ascertained[3] that the 9α-hydroxylation of pregnenolone by *C. sp.* is partly followed by 7α-hydroxylation of the 9α-hydroxy derivative formed, which results in a decrease in the yield of compound **1a**.

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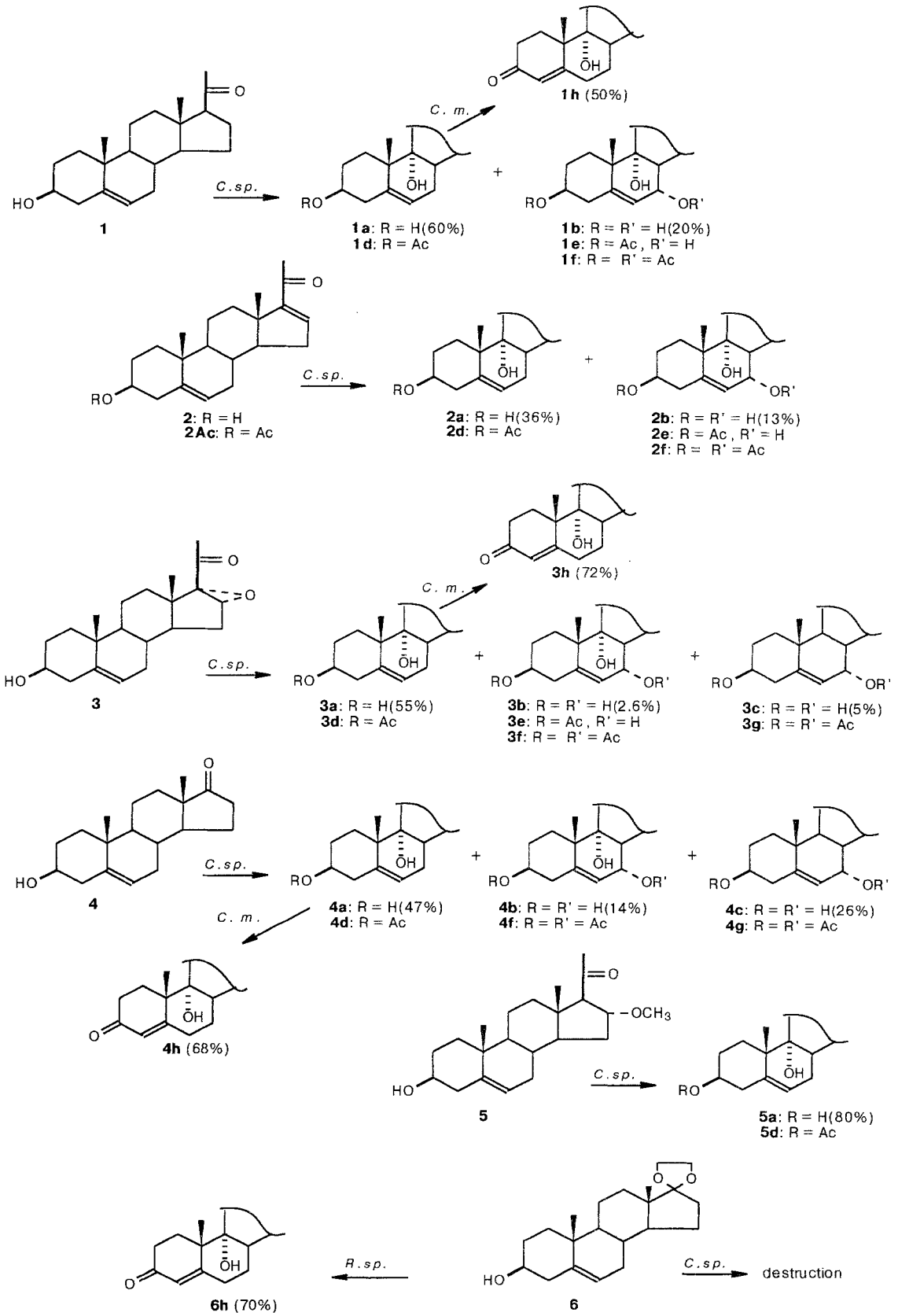
Scheme 1



Note. *C. sp.* — *Circinella sp.*, *R. sp.* — *Rhodococcus sp.*, *C. m.* — *Corynebacterium mediolanum.*

Table 1. Transformation of pregnenolone **1** by the growing (in the wort) or removed mycelium of *Circinella sp.*

Transformation medium	pH	Steroid contents after 24 h of transformation (%)			
		Starting substrate **1**	9α-OH **1a**	7α,9α-$(OH)_2$ **1b**	Total amount of steroids
Brewer's wort	5.0	32	50	10	96
Phthalate buffer	4.0	44	26	8	80
Acetate buffer	5.3	95	—	—	95
Phosphate buffer	6.8	28	38	8	90
Tap water	—	16	38	14	86

Therefore, it was necessary to elucidate the conditions under which the process of the hydroxylation of steroid substates by the *C. sp.* culture takes place.

First we studied the transformation of pregnenolone **1** by growing mycelium of *C. sp.* and by *C. sp.* removed from the medium, each suspended either in water or in different buffers (Table 1). The most active process of pregnenolone **1** transformation into 9α-hydroxy derivative **1a** (50 % yield) is performed by growing mycelium. The removed mycelium was considerably less active (38 % yield of **1a** in phosphate buffer or in water). The mycelium suspended in acetate buffer manifested the lowest capacity for transforming steroids. We have not yet found the reasons for the inhibition of the steroid-hydroxylase system of *C. sp.* in acetate buffer.

The incubation of pregnenolone 3β-acetate (**1Ac**) and 16-dehydroxypregnenolone 3β-acetate (**2Ac**) showed *C. sp.* to reveal a weak deacetylating activity, since **1Ac** and **2Ac** transformations, unlike those of **1** and **2**, proceed very slowly (72 h) leading to the corresponding 9α-hydroxy derivatives in lesser yields: 48 and 28 % for 9α-hydroxy derivatives formed by the transformation of **1** and **1Ac**,[3] and 36 and 27 % for those formed from **2** and **2Ac**, respectively.

Since steroid **2** is mostly available precisely in the form of 3β-acetate **2Ac** and the chemical method of its hydrolysis appears to be ineffective and prolonged (the hydrolysis of the 3β-acetyl group after several days of refluxing with $KHCO_3$ in dioxane is accompanied by considerable tar formation and yields only 42 % of **2**), we carried out the hydrolysis of **2Ac** using the *Arthrobacter citreus* bacteria. When starting content of the steroid substrate in the culture fluid is 2 g/L, the bacterial deacetylation proceeds in 47 h at 30 °C leading to hydroxy compound **2** in 85 % yield.

We studied the dynamics of the accumulation of Δ^5-3β-hydroxysteroid **1**, 16α,17α-epoxypregnenolone (**3**), and androstenolone (**4**) transformation products (Fig. 1). Figure 1 demonstrates that the maximum accumulation of 9α-hydroxy derivatives **1a** and **4a** was observed after 24 h of transformation of compounds **1** and **4** (Fig. 1, *a*, *c*), whereas the peak accumulation of epoxide **3a** took place after 30 h of transformation (Fig. 1, *b*). The transformation of the C-21 steroids **1** and **3** and of the C-19 steroid **4** differ in the rates of formation of the side 7α-hydroxy products. 9α- and 7α-hydroxylation of C-19 steroid **4** start simultaneously, but the introduction of the 7α-hydroxy group proceeds at lower rate (Figure 1, *c*). The side 7α-hydroxylation of C-21 steroids **1** and **3**, as compared to that of **4**, is characterized by a more prolonged lag phase, but it also proceeds at a rate lower than that of 9α-hydroxylation.

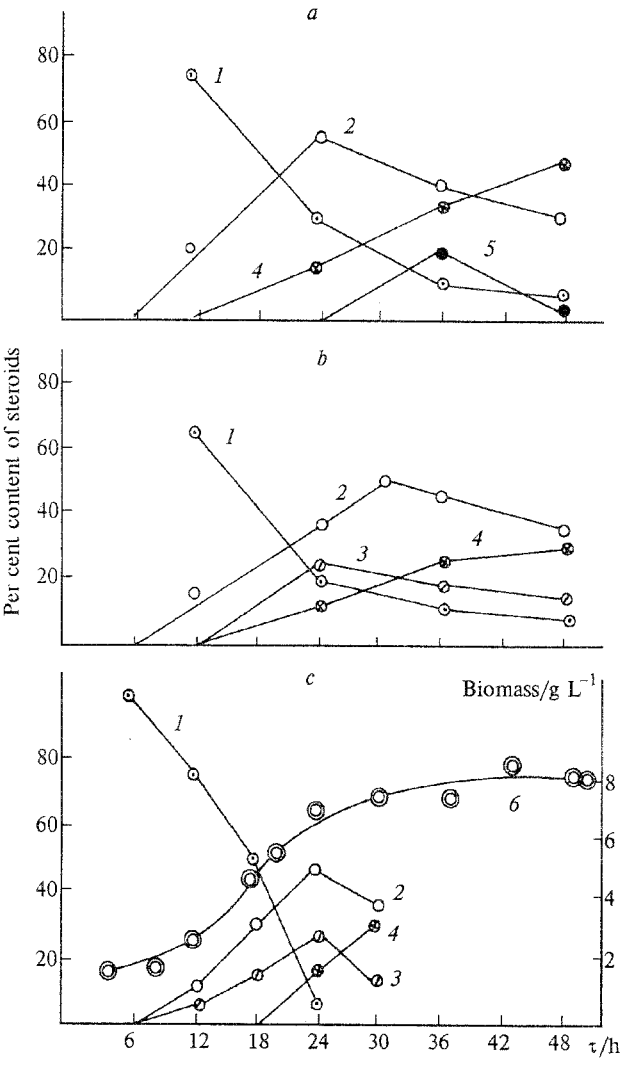


Figure 1. Dynamics of accumulation of the products of the transformation of pregnenolone **1** (*a*), 16α,17α-epoxypregnenolone **3** (*b*), and androstenolone **4** (*c*) by *Circinella sp. IOKh-1220* culture. *1*, the original substrate; *2*, 9α-hydroxy derivative; *3*, 7α-hydroxy derivative; *4*, 7α,9α-dihydroxy derivative; *5*, unidentified product (during the transformation of **1**); *6*, biomass.

Table 2. Dependance of the hydroxylation of pregnenolone **1** on the age of the *Circinella sp.* culture and on the starting amount of the steroid

Age mycelium/h	Starting contents of steroid substrate/g L^{-1}	Specific activity μmol g^{-1} h^{-1}	Steroid contents after 24 h of transformation (%)			
			Starting substrate **1**	9α-OH **1a**	7α,9α-$(OH)_2$ **1b**	Total amount of steroids
8	0.5	12.7	8	36	24	84*
16	0.5	9.5	12	60	20	92
24	0.5	6.6	29	50	16	95
24	1.0	6.2	26	32	18	76
32	0.5	5.8	24	48	12	84

*Including 16 % of an unidentified product

Since 17-ketone **4** is more intensively hydroxylated than are C-21 steroids, its 7α-monohydroxy derivative (**4c**) is formed in 26 % yield, whereas the transformation of **3** affords only a 5 % yield of its 7α-hydroxy analog (**3c**), while with compounds **1** and **2** the corresponding 7α-hydroxy derivatives do not accumulate at all. It should be noted that the overall rate of the modification of C-19 steroids is higher than that of C-21 steroids. Thus, by the incubation of androstenolone with *C. sp.* the almost complete disappearance of the former from the reaction mixture is observed already after 24 h, while in the case of C-21 steroids **1** and **3**, as well as in the case of diene **2** (which is not displayed in Fig. 1), there are up to 10 % of the initial substrates remaining in the culture fluid even after 40 h of transformation. By this time, the amount of the side 7α,9α-dihydroxy derivatives accumulated reaches 40 %. The analysis of the data presented shows that to obtain the target 9α-hydroxy analogs of Δ^5-3β-hydroxysteroids of the pregnane series in the maximal yields, their transformation should be interrupted before the complete disappearance of the starting substrate.

Reasoning from the literature data,[2,4] we suggest that *C. sp.* carries out both 9α- and 7α-hydroxylation by the action of only one enzyme and not two, as do the other dihydroxylating cultures, *e.g.*, *Aspergillus niger*. This fungus performs the side 6β-hydroxylation only after the appearance of the main hydroxy derivative, which is considered the inductor of 6β-hydroxylase.[2] *A. niger* forms only the 11α,6β-dihydroxy compound as a side product of the transformation, but it never forms the 6β-monohydroxy compound alone.[2] In the case of *C. sp.*, the possibility of the formation of 9α- or 7α-hydroxy derivatives is most likely to be determined by the character of enzyme-steroid binding. In this connection, it should be particularly noted that the example with *C sp.* provides a good confirmation of Jones'[5,6] hypothesis concerning enzyme-substrate complex formation and the orientation of the hydroxy groups introduced. This hypothesis is discussed in detail in ref. 4. In fact, *C. sp.* is capable of the effective formation of 9α-hydroxy analogs of androstenolone, *i.e.*, of compounds with terminal oxygen functions (3-OH and 17-CO) and also of pregnane compounds with 3-OH and 20-CO groups, the fragments mentioned allowing the greatest possible binding between the enzyme and the substrate. However, when incubated for 72 h with a substrate having only one oxygen function available for binding, *i.e.*, with compound **6**, *C. sp.* does not perform 9α-hydroxylation (Scheme 1).

It is of interest to compare the data obtained with the results of the transformation of androstenolone ethyleneketal **6** by the 9α-hydroxylating bacteria *Rhodococcus sp. IOKh-77*, which easily transform **6** into 9α-hydroxy-Δ^4-3-keto derivative (**6h**) in 70 % yield (16 h; concentration of **6** in culture fluid 0.5 g/L).* One can suggest that the mechanism of the bacterial 9α-hydroxylation of Δ^4-3-ketosteroids differs from that of 9α-hydroxylation by fungi. This suggestion is confirmed by the fact that cobalt salts, for instance, are inhibitors of bacterial 9α-hydroxylation but increase the yield of 9α-hydroxy derivatives (from 19 up to 49 %) in the transformation of 5α(H)-androstanes by *Absidia regnieri* fungi.[6]

In the next stage of the work we studied the effects of the age of the mycelium and the amount of steroid applied on the yield of the 9α-monohydroxy derivative. From the data listed in Table 2 it follows that the maximum specific activity was demonstrated by the mycelium, when it was used for the transformation at the age of 8 h, that is, in the beginning of the phase of logarithmic growth (Fig. 1, *c*). However, the maximum yield of the target 9α-hydroxypregnenolone **1a** with a small amount (12 %) of the starting material left and a low intensity of steroid metabolite degradation (92 % overall yield of the reaction products) was obtained, when 16 h-old mycelium was used (the end of the phase of logarithmic growth), provided the initial contents of the steroid added to the culture fluid did not exceed 0.5 g/L. Increasing the amount of pregnenolone **1** to 1 g/L enhanced the destructive processes and lowered the overall yield from 95 to 76 %, and decreased the yield of 9α-hydroxy analog **1a** from 50 to 32 % (Table 2).

The optimal conditions of the transformation of pregnenolone **1**, *i.e.*, the transformation of 0.5 g/L of the substrate by 16 h-old mycelium growing in brewer's

*The substrate with both carbonyl groups unavailable for the enzyme, *e.g.*, progesterone bisethyleneketal, is not amenable to transformation by *Rhodococcus sp.*

wort, were used to obtain 9α-hydroxy derivatives of compounds **3**,**4**, and 16α-methoxy pregnenolone (**5**) in 55, 47, and 80 % yield, respectively. In the process, the 16α-methoxy group entirely blocked the side 7α-hydroxylation. Unfortunately, the 9α-monohydroxylation of 16-dehydropregnenolone **2** under similar conditions occured only in 36 % yield, the amount of the side product, 7α,9α-dihydroxy derivative (**26**), formed, though, was also rather small (13%). Consequently, the intensification of the transformation of **2** into **2a** needs a somewhat different approach.

The structures of all of the compounds obtained were fully confirmed by physicochemical data. The presence of the 9α-hydroxy group was deduced from the downfield shift of the signal for the angular 19-methyl group protons in the ^{1}H NMR spectra (0.1—0.12 ppm for androstanes and 0.14—0.15 ppm for pregnanes). Futhermore, additional confirmation of the structures was provided by the transformation of a number of 9α-hydroxy compounds obtained into the known compounds (**1a**, **3a**, **4a** into **1h**, **3h**, **4h**, correspondingly). The introduction of a 7-hydroxy group was also unequivocally demonstrated by the ^{1}H NMR spectra. The very characteristic manifestation of the proton at C-6 in 7-hydroxy isomers is known.[7,8] When the 7α-OH group appears in a molecule, the signal for 6-H moves downfield approximately 0.25—0.3 ppm, whereas in 7β-isomers the corresponding signal moves upfield 0.07 ppm. The vicinal coupling constants $J_{6,7}$ of 7α- and 7β-OH isomers are also different (5 Hz and 2.5 Hz, respectively).[9] The spectral data obtained by us are in entire agreement with the literature data.

Additional proof of the stucture of the 7-hydroxy compounds was provided by their acylation, though we ran into some difficulties with it. The 3β-hydroxy group is well known to be acylated rather easily by acetic anhydride in pyridine. The reaction usually proceeds in 18—20 h, which was exactly the case with the transformation of every one of the five compounds **1a**—**5a** into their 3β-acetates (**1d**—**5d**). The acylation of the 7α-hydroxy group under these conditions proceeds much more slowly. Thus, one needs 48 h to obtain the diacetate **4g** from its precursor, androst-5-ene-3β,7α-diol-17-one (**4c**); the completion of the analogous transformation of 16α,17α-epoxide (**3c** → **3g**) occurs only after 96 h.

The simultaneous introduction of two sterically close hydroxy groups into the 9α- and 7α-positions of the same molecule probably results in their interaction, making acylation of the 7α-hydroxy group all the more difficult. Even 7 days is not enough for more than 50 % acylation of **1b**. We were able to obtain 3β,7α-diacetates **2f** and **3f** only using 4-dimethylaminopyridine as a catalyst.

Experimental

The transformation products were analyzed using methods of analytical and preparative chromatography on Silufol US-254 and silica gel 40/100 μ. The chromatograms were developed by heating with a 1 % solution of $Ce(SO_4)_2$ in 10 % H_2SO_4.

Melting points were determined using an electrically heated block. IR spectra (ν/cm^{-1}) were obtained on a Specord M-80 spectrometer in $CHCl_3$. Mass spectra (*m/e*) were recorded on a Varian MAT CH-6 instrument. ^{1}H NMR spectra (δ, ppm) of all of the compounds obtained were recorded on a Bruker WM-250 instrument in $CDCl_3$.

The microbial transformations of the steroids were carried out using the microorganisms *Circinella sp.*, *Arthrobacter citreus*, and *Corynebacterium mediolanum*. The spores of *Circinella* fungi from the surface of an agar medium were added to 50 mL of the hopless brewer's wort 7 °Blg at pH 6.0—6.5 in 250 mL Erlenmeyer flasks. The inoculate was grown on a shaker (200 rpm) at 26 °C for 70 h, then moved in 10 % (by volume) portions into flasks with the same medium for cultivation of the mycelium for carrying out the transformations. For the preparative transformation of pregn-5-en-3β-ol-20-one (**1**), pregna-5,16-dien-3β-ol-20-one (**2**), 16α,17α-epoxypregn-5-en-3β-ol-20-one (**3**), androst-5-en-3β-ol-17-one (**4**), 16α-methoxypregn-5-en-3β-ol-20-one (**5**), and of compound **4** ethyleneketal (**6**) the mycelium was grown for 16 h; for the determination of the conditions of the transformation of compounds **1**,**3**, and **4** — for 8—32 h. The steroid was added to the culture liquid in amounts of 0.5g/L as a solution in DMF, the concentration of the latter in the medium accounting for 2 %. The transformation was carried out for 6—72 h at 28 °C, while the medium was stirred at 200 rpm. Transformation time depended on the structure of the starting substrate and the experimental conditions. To investigate the 9α-hydroxylating activity of *C. sp.* in buffer solutions, 24 h-old mycelium was removed from the medium, washed with a physiological solution and resuspended in an equal amount of water or 0.05 M buffer.

The amount of biomass was estimated from dry mycelium weight. The specific activity of the mycelium was expressed as the quantity of micromoles of steroid transformed by 1 g of biomass in 1 h.

The conditions under which the inoculum of *A. citreus*, *C. mediolanum*, and *Rhodococcus IOKh-77* were prepared, as well as the conditions of the transformation of 3β-acetate **2Ac** and compounds **3a**, **4a**, and **6** by the cultures mentioned, were similar to those described in Refs. 10, 11, 12, respectively. The products of the transformations by *C. sp.* fungi, as well as by the above-named bacteria, were extracted from the culture fluid with chloroform. The extracts were dried over $MgSO_4$, and the solvent was evaporated *in vacuo*.

3β,9α-Dihydroxypregn-5-en-20-on (1a)[3] (0.12 g) was isolated from the reaction mixture obtained after the transformation of **1** (0.2 g) by *C. sp.* for 24 h. Along with **1a**, 0.024 g of starting substrate **1** and 0.04 g of 3β,7α,9α-trihydroxypregn-5-en-20-one (**1b**)[3] were isolated.

3β-Acetoxy-9α-hydroxypregn-5-en-20-one (1d). Pyridine (0.3 mL) and Ac_2O (0.15 mL) were added to **1a** (0.03 g). Afted 18 h the liquids were entirely evaporated. Crystallization afforded 0.02 g of **1d**, m.p. 234—236 °C ($CHCl_3$—C_6H_{14}). IR: 1035, 1365, 1378, 1700, 1730, 3570. ^{1}H NMR: 0.6 (s, 3 H, Me-18); 1.13 (s, 3 H, Me-19); 2.01 (s, 3 H, OAc(3)); 2.1 (s, 3 H, Me-21); 4.55 (m, 1 H, HC(3)); 5.41 (broad s, 1 H, HC(6)). MS: 314 $[M-CH_3COOH]^+$, 296 $[M-CH_3COOH-H_2O]^+$, 281 $[M-CH_3COOH-H_2O-CH_3]^+$, 253 $[M-CH_3COOH-H_2O-COCH_3]^+$.

3β-Acetoxy-7α,9α-dihydroxypregn-5-en-20-one (1e). A solution of **1b** (0.03 g) in pyridine (1.4 mL) and Ac_2O (0.3 mL) was maintained at ~20 °C for 7 days. Then the solution was

quenched with cold water, and the precipitate was filtered, washed and dried. Preparative chromatography on a SiO_2 layer (Me_2CO—C_6H_{14}) afforded *a*) 0.015 g of **1e** m.p. 201—214 °C ($CHCl_3$—C_6H_{14}). {IR: 1035, 1370, 1380, 1705, 1730, 3490, 3610. ^{1}H NMR: 0.67 (s, 3 H, Me-18); 1.13 (s, 3 H, Me-19); 2.05 (s, 3 H, OAc(3)); 2.17 (s, 3 H, Me-21); 3.95 (broad s, 1 H, HC(7)); 4.68 (m, 1 H, HC(3)); 5.65 (dd, 1 H, HC(6)). MS: 330 $[M-CH_3COOH]^+$, 312 $[M-CH_3COOH-H_2O]^+$, 294 $[M-CH_3COOH-2H_2O]^+$, 279 $[M-CH_3COOH-2H_2O-CH_3]^+$, 251 $[M-CH_3COOH-2H_2O-COCH_3]^+$};

b) 0.015 g of **3β,7α-diacetoxy-9α-hydroxypregn-5-en-20-one (1f)**, m.p. 182—186 °C ($CHCl_3$—C_6H_{14}) {IR: 1019, 1040, 1375, 1705, 1732, 3583. ^{1}H NMR: 0.65 (s, 3 H, Me-18); 1.13 (s, 3 H, Me-19); 2.04 (s, 3 H, OAc(3)); 2.09 (s, 3 H, OAc(7)); 2.15 (s, 3 H, Me-21); 4.66 (m, 1 H, HC(3)); 5.15 (broad s, 1 H, HC(7)); 5.6 (dd, 1 H, HC(6)). MS: 372 $[M-CH_3COOH]^+$, 354 $[M-CH_3COOH-H_2O]^+$, 312 $[M-2CH_3COOH]^+$, 294 $[M-2CH_3COOH-2H_2O]^+$, 279 $[M-2CH_3COOH-H_2O-CH_3]^+$}.

9α-Hydroxypregn-4-ene-3,20-dione (1h). The transformation of 9α-hydroxypregnenolone **1a** (0.03 g) by *C. mediolanum* as described earlier[11] gave 0.016 g of **1h**, m.p. 187—190 °C ($CHCl_3$—C_6H_{14}), showing no depression of the melting point in a mixed probe with the known sample[12].

3β,9α-Dihydroxypregna-5,16-dien-20-one (2a). The reaction mixture obtained after the transformation of **2** (0.5 g) by *C. sp.* for 24 h was separated by TLC (Me_2CO—C_6H_{14}, 2:3). Along with 0.075 g of **2**, the separation afforded: *a*) 0.18 g of **2a**, m.p. 227—230 °C (from MeOH—H_2O) {IR: 1050, 1375, 1585, 1663, 3610. ^{1}H NMR: 0.95 (s, 3 H, Me-18); 1.2 (s, 3 H, Me-19); 2.27 (s, 3 H, Me-21); 3.5 (m, 1 H, HC(3)); 5.43 (t, 1 H, HC(6)); 6.73 (d, 1 H, HC(16)). MS: 330 M^+, 312 $[M-H_2O]^+$, 294 $[M-2H_2O]^+$, 279 $[M-2H_2O-Me]^+$}; *b*) 0.063 g of **3β,7α,9α-trihydroxypregna-5,16-dien-20-one (2b)**, m.p. 230—235 °C (decomp.)[13] (from $CHCl_3$—C_6H_{14}) {IR: 1045, 1585, 1665, 3610. ^{1}H NMR: 0.96 (s, 3 H, Me-18); 1.16 (s, 3 H, Me-19); 2.3 (s, 3 H, Me-21); 3.6 (m, 1 H, HC(3)); 3.9 (broad s, 1 H, HC(7)); 5.63 (dd, 1 H, HC(6)); 6.76 (broad s, 1 H, HC(16)). MS: 328 $[M-H_2O]^+$, 310 $[M-2H_2O]^+$, 292 $[M-3H_2O]^+$}.

II. Analogously, from the reaction mixture obtained after 70 h of transformation of **2Ac** (0.1 g) with *C. sp.* 0.005 g of **2Ac**, 0.005 g of **2**, 0.025 g of **2a**, and 0.013 g of **2b** were isolated.

3β-Acetoxy-9α-hydroxypregna-5,16-dien-20-one (2d), m.p. 178—181 °C (MeOH—H_2O), was isolated in the amount of 0.023 g after the acetylation of **2a** (0.027 g). IR: 1035, 1210, 1250, 1715, 1725, 3580. ^{1}H NMR: 0.92 (s, 3 H, Me-18); 1.2 (s, 3 H, Me-19); 2.05 (s, 3 H, OAc(3)); 2.26 (s, 3 H, Me-21); 4.59 (m, 1 H, HC(3)); 5.45 (t, 1 H, HC(6)); 6.72 (m, 1 H, HC(16)). MS: 312 $[M-CH_3COOH]^+$, 294 $[M-CH_3COOH-H_2O]^+$, 249 $[M-CH_3COOH-H_2O-CH_3]^+$.

3β-Acetoxy-7α,9α-hydroxypregna-5,16-dien-20-one (2e). The acetylation of **2b** (0.05 g) by Ac_2O in Py for 5 days afforded 0.027 g of **2e**, m.p. 208—212 °C (EtOAc—C_6H_{14}). IR: 1035, 1245, 1590, 1650, 1728, 3400. ^{1}H NMR: 0.94 (s, 3 H, Me-18); 1.15 (s, 3 H, Me-19); 2.05 (s, 3 H, OAc(3)); 2.29 (s, 3 H, Me-21); 3.92 (broad s, 1 H, HC(7)); 4.68 (m, 1 H, HC(3)); 5.66 (broad s, 1 H, HC(6)), 6.79 (s, 1 H, HC(16)). MS: 310 $[M-CH_3COOH-H_2O]^+$.

3β,7α-Diacetoxy-9α-hydroxypregna-5,16-dien-20-one (2f). **2e** (0.025 g) was acetylated under the usual conditions with the addition of a few crystals of 4-dimethylaminopyridine (4-DMAP) to give 0.022 g of diacetate **2f** (oil). IR: 1035, 1245, 1370, 1585, 1665, 1730, 3580. ^{1}H NMR: 0.95 (s, 3 H, Me-18); 1.16 (s, 3 H, Me-19); 2.05 (s, 3 H, OAc(3)); 2.09 (s, 3 H, OAc(7)); 2.28 (s, 3 H, Me-21); 4.67 (m, 1 H, HC(3)); 5.23 (broad s, 1 H, HC(7)); 5.6 (broad s, 1 H, HC(6)), 6.71 (s, 1 H, HC(16)).

3β,9α-Dihydroxy-16α,17α-epoxypregn-5-en-20-one (3a). Crystallization of the dry residue obtained after the transformation of **3** (0.5 g) by *C sp.* for 26 h yielded 0.275 g of **3a**, m.p. 221—223 °C (EtOAc—C_6H_{14}). IR: 1050, 1260, 1380, 1700, 3600. ^{1}H NMR: 1.04 (s, 3 H, Me-18); 1.15 (s, 3 H, Me-19); 2.04 (s, 3 H, Me-21); 3.51 (m, 1 H, HC(3)); 3.68 (s, 1 H, HC(16)); 5.39 (d, 1 H, HC(16)). MS: 328 $[M-H_2O]^+$, 313 $[M-H_2O-Me]^+$. The workup of the mother liquor afforded: *a)* 0.013 g of **3β,7α,9α-trihydroxy-16α,17α-epoxypregn-5-en-20-one (3b)**, m.p. 252—257 °C (decomp.) (EtOAc—C_6H_{14}). {IR: 1050, 1380, 1705, 3620. ^{1}H NMR: 1.08 (s, 3 H, Me-18); 1.13 (s, 3 H, Me-19); 2.06 (s, 3 H, Me-21); 3.63 (m, 1 H, HC(3)); 3.76 (s, 1 H, HC(16)); 3.84 (broad s, 1 H, HC(7)); 5.65 (d, 1 H, HC(6)). MS: 362 M^+, 344 $[M-H_2O]^+$, 326 $[M-2H_2O]^+$, 311 $[M-2H_2O-Me]^+$, 293 $[M-3H_2O-Me]^+$}; *b*) 0.025 g of **3β,7α-dihydroxy-16α,17α-epoxypregn-5-en-20-one (3c)**, m.p. 195—200 °C (EtOAc—C_6H_{14}). {IR: 1050, 1210, 1380, 1700, 3600, 3660. ^{1}H NMR: 1.02 (s, 3 H, Me-18); 1.07 (s, 3 H, Me-19); 2.05 (s, 3 H, Me-21); 3.56 (m, 1 H, HC(3)); 3.72 (s, 1 H, HC(16)); 3.8 (broad s, 1 H, HC(7)); 5.61 (dd, 1 H, HC(6)). MS: 346 M^+, 328 $[M-H_2O]^+$, 313 $[M-H_2O-Me]^+$, 295 $[M-2H_2O-Me]^+$, 285 $[M-H_2O-COCH_3]^+$, 267 $[M-2H_2O-COCH_3]^+$}.

3β-Acetoxy-16α,17α-epoxy-9α-hydroxypregn-5-en-20-one (3d). The acetylation of **3a** (0.021 g) gave 0.022 g of **3d**, m.p. 187—189 °C (MeOH—H_2O). IR: 1035, 1260, 1380, 1710, 1732, 3590. ^{1}H NMR: 1.06 (s, 3 H, Me-18); 1.18 (s, 3 H, Me-19); 2.04 (s, 6 H, Me-21 and OAc(3)); 3.68 (s, 1 H, HC(16)); 4.57 (m, 1 H, HC(3)); 5.42 (broad s, 1 H, HC(6)). MS: 328 $[M-CH_3COOH]^+$, 310 $[M-CH_3COOH-H_2O]^+$, 295 $[M-CH_3COOH-H_2O-CH_3]^+$, 285 $[M-CH_3COOH-COCH_3]^+$.

3-Acetoxy-7α,9α-dihydroxy-16α,17α-epoxypregn-5-en-20-one (3e). **3b** (0.05 g) was acetylated for 4 days by the standard method. The workup yielded 0.048 g of monoacetate **3e**, m.p. 210—216 °C (EtOAc—C_6H_{14}). IR: 1035, 1260, 1370, 1715, 3500, 3620. ^{1}H NMR: 1.07 (s, 3 H, Me-18); 1.12 (s, 3 H, Me-19); 2.04 (s, 3 H, OAc(3)); 2.05 (s, 3 H, Me-21), 3.75 (s, 1 H, HC(16)); 3.82 (broad s, 1 H, HC(7)); 4.67 (m, 1 H, HC(3)); 5.65 (d, 1 H, HC(6)). MS: 344 $[M-CH_3COOH]^+$, 326 $[M-CH_3COOH-H_2O]^+$, 308 $[M-CH_3COOH-2H_2O]^+$, 283 $[M-CH_3COOH-H_2O-COCH_3]^+$, 265 $[M-CH_3COOH-2H_2O-COCH_3]^+$.

3β,7α-Diacetoxy-16α,17α-epoxy-9α-hydroxypregn-5-en-20-one (3f). 0.1 mL of Ac_2O and a few crystals of 4-DMAP were added to a solution of acetate **3e** (0.04 g) in Py (1 ml) and the mixture was allowed to sit for 24 h. The usual workup afforded 0.016 g of **3f**, m.p. 223—226 °C (EtOAc—C_6H_{14}). IR: 1018, 1040, 1210—1250, 1375, 1700, 1725, 3570. ^{1}H NMR: 1.07 (s, 3 H, Me-18); 1.15 (s, 3 H, Me-19); 2.05 (s, 6 H, OAc(3) and Me-21); 2.12 (s, 3 , OAc(7)), 3.71 (s, 1 H, HC(16)); 4.66 (m, 1 H, HC(3)); 5.12 (broad s, 1 H, HC(7)); 5.57 (d, 1 H, HC(6)). MS: 326 $[M-2CH_3COOH]^+$.

3β,7α-Diacetoxy-16α,17α-epoxypregn-5-en-20-one (3g). The acetylation of **3c** (0.027 g) by Ac_2O in Py for 4 days yielded 0.02 g of **3g**, m.p. 190—195 °C (EtOAc—C_6H_{14}). IR: 1035, 1250—1265, 1380, 1710, 1730. ^{1}H NMR: 1.04 (s,

3 H, Me-18); 1.07 (s, 3 H, Me-19); 2.04 (s, 6 H, OAc(3) and Me-21); 2.07 (s, 3 H, OAc(7)), 3.69 (s, 1 H, HC(16)); 4.67 (m, 1 H, HC(3)); 4.96 (t, 1 H, HC(7)); 5.57 (d, 1 H, HC(6)). MS: 370 $[M-CH_3COOH]^+$, 327 $[M-CH_3COOH-COCH_3]^+$, 310 $[M-2CH_3COOH]^+$, 295 $[M-2CH_3COOH-CH_3]^+$, 267 $[M-2CH_3COOH-COCH_3]^+$.

The transformation of **3a** (0.025 g) by *C. mediolanum* for 17 h gave 0.018 g of diketone **3h**, m.p. 228—229 °C (EtOAc—C_6H_{14}) showing no depression of the melting point in a mixed probe with the known sample.[14]

3β,9α-Dihydroxyandrost-5-en-17-one (4a). Compound **4** (0.5 g) was transformed by *C. sp.* for 22 h. The reaction mixture was separated by TLC (Me_2CO—C_6H_{14}, 2:3) to afford, besides 0.25 g of compound **4**, *a*) 0.24 g of compound **4a**, m.p. 184—187 °C (EtOAc—C_6H_{14}) {IR: 1038, 1050, 1735, 3610. ^1H NMR: 0.88 (s, 3 H, Me-18); 1.17 (s, 3 H, Me-19); 3.52 (m, 1 H, HC(3)); 5.45 (broad s, 1 H, HC(6)). MS: 304 M^+, 286 $[M-H_2O]^+$, 268 $[M-2H_2O]^+$, 253 $[M-2H_2O-Me]^+$}; *b*) 0.078 g of **3β,7α,9α-trihydroxyandrost-5-en-17-one (4b)**, m.p. 234—236 °C (EtOAc—C_6H_{14}). {IR: 1040, 1738, 3620. ^1H NMR: 0.93 (s, 3 H, Me-18); 1.15 (s, 3 H, Me-19); 3.65 (m, 1 H, HC(3)); 4.03 (broad s, 1 H, HC(7)), 5.68 (d, 1 H, HC(6)) MS: 302 $[M-H_2O]^+$, 284 $[M-2H_2O]^+$, 266 $[M-3H_2O]^+$}; *c*) 0.137 g of **3β,7α-dihydroxyandrost-5-en-17-one (4c)**, m.p. 183—187 °C (EtOAc—C_6H_{14}).[15]

3β-Acetoxy-9α-hydroxyandrost-5-en-17-one (4d). The acetylation of compound **4a** (0.043 g) under normal conditions (Ac_2O in Py, 18 h) afforded 0.04 g of 3-acetate **4d**, m.p. 173—174 °C (EtOAc—C_6H_{14}). IR: 1035, 1255, 1375, 1735, 3570. ^1H NMR: 0.89 (s, 3 H, Me-18); 1.18 (s, 3 H, Me-19); 2.04 (s, 3 H, OAc(3)), 4.58 (m, 1 H, HC(3)); 5.47 (broad s, 1 H, HC(6)). MS: 286 $[M-CH_3COOH]^+$, 271 $[M-CH_3COOH-CH_3]^+$, 268 $[M-CH_3COOH-H_2O]^+$.

3β,7α-Diacetoxy-9α-hydroxyandrost-5-en-17-one (4f). The acetylation of **4a** (0.08 g) by Ac_2O in Py proceeded for 5 days. Isolation by TLC (Me_2CO—C_6H_{14}, 2:3) yielded 0.062 g of **4f**, m.p. 176—177 °C (EtOAc—C_6H_{14}). IR: 1020, 1035, 1205—1235, 1375, 1735, 3580. ^1H NMR: 0.9 (s, 3 H, Me-18); 1.16 (s, 3 H, Me-19); 2.07 (s, 3 H, OAc(3)), 2.11 (s, 3 H, OAc(7)), 4.68 (m, 1 H, HC(3)); 5.29 (broad s, 1 H, HC(7)), 5.64 (dd, 1 H, HC(6)). MS: 344 $[M-CH_3COOH]^+$, 326 $[M-CH_3COOH-H_2O]^+$, 284 $[M-2CH_3COOH]^+$ 266 $[M-2CH_3COOH-H_2O]^+$.

3β,7α-Diacetoxyandrost-5-en-17-one (4g). The acetylation of **4c** (0.05 g) under normal conditions (Ac_2O in Py) for 2 days afforded 0.027 g of **4g**, m.p. 161—166 °C (EtOAc—C_6H_{14}).[16]

9α-Hydroxyandrost-4-en-3,17-dione (4h). **4a** (0.025 g) was transformed by the above-described method using *C. mediolanum* to give 0.017 g of 3-ketone **4h**, m.p. 221—223 °C ($CHCl_3$—C_6H_{14}), showing no depression of the melting point in a mixed probe with the known sample[12].

3β,9α-Dihydroxy-16α-methoxypregn-5-en-20-one (5a). Crystallization of the dry residue obtained after the transformation of compound **5** (0.42 g) by *C. mediolanum* for 30 h gave 0.396 g of diol **5a**, m.p. 204—206 °C (MeOH—H_2O). IR: 1050, 1095, 1703, 3575, 3600. ^1H NMR: 0.65 (s, 3 H, Me-18); 1.13 (s, 3 H, Me-19); 2.18 (s, 3 H, Me-21), 3.22 (s, 3 H, MeO(16)), 3.52 (m, 1 H, HC(3)); 4.36 (t, 1 H, HC(16)), 5.42 (d, 1 H, HC(6)). MS: 344 $[M-H_2O]^+$, 330 $[M-CH_3OH]^+$, 312 $[M-H_2O-CH_3OH]^+$, 294 $[M-2H_2O-CH_3OH]^+$, 279 $[M-2H_2O-CH_3OH-CH_3]^+$.

3-Acetoxy-9α-hydroxy-16α-methoxypregn-5-en-20-one (5d). The acetylation of diol **5a** (0.16 g) for 18 h afforded 0.175 g of 3-acetate **5d**. The crystalization yielded 0.127 g of monoacetate **5d**, m.p. 175—176 °C (EtOAc—C_6H_{14}). IR: 1038, 1092, 1255, 1375, 1700, 1725, 3580. ^1H NMR: 0.64 (s, 3 H, Me-18); 1.15 (s, 3 H, Me-19); 2.04 (s, 3 H, OAc(3)); 2.19 (s, 3 H, MeO(21)); 3.22 (s, 3 H, MeO(16)); 4.36 (t, 1 H, HC(16)); 4.59 (m, 1 H, HC(3)); 5.44 (d, 1 H, HC(6)). MS: 354 $[M-CH_3OH-H_2O]^+$, 344 $[M-CH_3COOH]^+$, 326 $[M-CH_3COOH-H_2O]^+$, 312 $[M-CH_3COOH-CH_3OH]^+$, 294 $[M-CH_3OH-H_2O-CH_3COOH]^+$.

17,17-Ethylenedioxy-9α-hydroxyandrost-4-en-3-one (6h). The reaction mixture obtained after the transformation of ethyleneketal **6** (0.1 g) by *Rhodococcus sp.* for 16 h was separated by TLC (Me_2CO—C_6H_{14}, 1:3) to give 0.075 g of ketol **6h**, m.p. 216—218 °C (EtOAc—C_6H_{14}). IR: 1015, 1045, 1205, 1375, 1615, 1665, 3610. ^1H NMR: 0.91 (s, 3 H, Me-18); 1.33 (s, 3 H, Me-19); 3.9 (m, 4H, acetal(17)), 5.86 (d, 1 H, HC(4)). MS: 346 M^+, 328 $[M-H_2O]^+$, 384 $[M-C_2H_4(OH)_2]^+$.

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