STEROID DERIVATIVES. LXX.*

MICROBIAL HYDROXYLATION OF 17α,21-DIHYDROXY-16-METHYLENE-4-PREGNENE-3,20-DIONE

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Hydroxylation of compound *I* with known 11α-hydroxylating microorganisms has been investigated. While *Beauveria bassiana* gave the expected 11α-hydroxy derivative as the sole biotransformation product, one of the *Rhizopus nigricans* strains used afforded 7β-hydroxy compound *VII*, while the other produced a mixture of *VII* and the 6β-hydroxy analogue *X*. The structures of the products were demonstrated on the basis of their physical properties and by their chemical conversion to known derivatives.

In connection with our study on the preparation of corticoid-like compounds substituted in position 16 with a methylene group we also investigated the microbial 11α-hydroxylation of 17α,21-dihydroxy-16-methylene-4-pregnene-3,20-dione (*I*). From a series of known 11α-hydroxylators we chose *Beauveria bassiana* and two strains of *Rhizopus nigricans*, of which it is well known that they can perform the expected reaction with high yields and without the formation of a substantial amount of by-products.

Microbial hydroxylation of 16-methylene derivatives of the pregnane series is the subject of several papers. Usually, such compounds were transformed which were substituted in addition to position 16 also in the ring B[1-6]; such a substitution makes the hydroxylation of steroids with hydroxyl groups at positions 6 or 7, occurring often as by-products during 11α-hydroxylations, impossible. In all these instances the corresponding 11α-hydroxy derivative was isolated as the only biotransformation product. Similar results were observed when substance *I* was hydroxylated using *Fusarium* sp.[7], and its 1-dehydro derivative[7] or 21-deoxy derivative with the same microorganism[8] or by *Absidia orchidis*, *B. bassiana* and *R. nigricans*[9].

While during microbial hydroxylation of 16-methylene derivative *I* by *B. bassiana* a single product was obtained, as expected, *i.e.* 11α-hydroxy derivative *II*, two hydroxy

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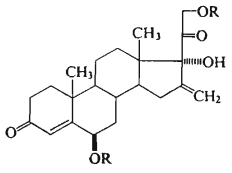
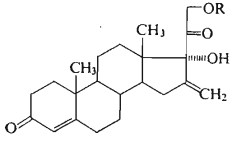
derivatives differing by their chemical and physical properties from 11α-hydroxy derivative *II* were obtained on incubation of *I* with two physiologically different strains of *Rhizopus nigricans*. The structure of substance *II* was proved both on the basis of its physical constants which agreed with those from the literature[7], and by the following chemical reactions. On partial acetylation of the trihydroxy com-



X, R = H
XI, R = Ac



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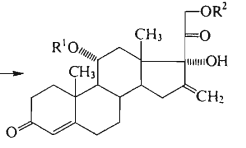
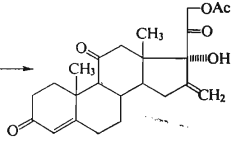
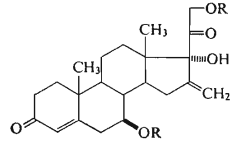
XII, R = Ac



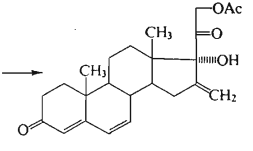
II, $R^1 = R^2 = H$
III, $R^1 = R^2 = Ac$
IV, $R^1 = H$; $R^2 = Ac$



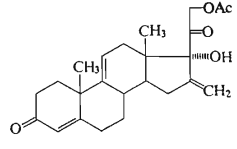
V



VII, R = H
V, R = Ac



IX



VI

pound *II* with a slight excess of acetic anhydride in pyridine at −20 to −40°C 21-monoacetate *IV** was obtained in a satisfactory yield. The monoacetate was converted on oxidation of the 11α-hydroxy group with chromium trioxide in conc. sulfuric acid to the 11-ketone *V* displaying a characteristic hypsochromic shift of its UV maximum at 237 nm, confirming thus the presence of a keto group in position 11 (ref.[10]). 21-Monoacetate *IV* was also transformed on mesylation of the 11α-hydroxy group and subsequent elimination of the methanesulfonic acid by sodium acetate in acetic acid to the known 21-acetoxy-17α-hydroxy-16-methylene-4,9(11)-pregnadiene-3,20-dione (*VI*) (ref.[11]) which was also prepared by another route. Acetylation of the trihydroxy compound *II* or of the 21-acetoxy derivative *IV* with acetic anhydride in pyridine under the usual conditions gave non-crystalline 11α,21-diacetate *III*.

The biotransformation of the compound *I* by a strain of *R. nigricans* from the collection of the Institute of Microbiology, Czechoslovak Academy of Sciences, led to product *VII* containing according to elemental analysis an additional hydroxy group, but different from the 11α-hydroxy derivative *II*. Compound *VII* was converted on acetylation with acetic anhydride in pyridine to diacetate *VIII* which gave on heating in acetic acid 21-acetoxy-17α-hydroxy-16-methylene-4,6-pregnadiene-3,20-dione (*IX*), displaying an UV absorption maximum at 285 nm, characteristic of substances containing a 3-keto-4,6-diene grouping in the molecule. The easy formation of this grouping may be explained only on the assumption that the hydroxy group in position 7 of compound *VII* was split off. The decrease of the UV maximum of diacetate *VIII* (ref.[10]) is also indicative of a hydroxy group at $C_{(7)}$. 7β-Configuration can be also inferred with certainty from the molecular rotation differences[10].

In contrast to the collection strain a freshly isolated strain of *R. nigricans* gave on hydroxylation of the starting compound *I* a mixture of two hydroxylation products, from which a small amount of the isomeric hydroxy derivative *X* was isolated in addition to the predominating 7β-hydroxy derivative *VII*. The distinct drop of the UV maximum of this compound (234 nm) indicated the presence of a hydroxy group at $C_{(6)}$[10]. The structure of compound *X* was proved by its conversion to diacetate *XI* which on reduction with zinc in acetic acid[12] gave the known 21-acetoxy-17α-hydroxy-16-methylene-4-pregnene-3,20-dione (*XII*) (ref.[11]). The 6β-configuration of the hydroxy group in compound *X* followed unambiguously from the molecular rotation difference[10].

The decisive role of the substitution of the steroid D-ring (especially at position 16) for the qualitative course of hydroxylation by common 11α-hydroxylators was demonstrated in the preceding paper[9] for progesterone type derivatives. In addition to other microorganisms we also employed *R. nigricans* and *B. bassiana*, which trans-

* The validity of these findings was confirmed by partial acetylation of 3β,17α,21-trihydroxy-5-pregnen-20-one and 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (epicortisol) under the same conditions; in both cases the corresponding 21-monoacetate was isolated as the predominant reaction product.

formed various 16,17-substituted derivatives always to substances with a hydroxy group in position 11α. Thus, for example, 17α-hydroxy-16-methylene-4-pregnene-3,20-dione, differing from the substrate *I* only by the absence of the 21-hydroxy group, gave on transformation with the mentioned microorganisms the corresponding 11α-hydroxy derivative[9] as the main product. 11α-Hydroxy derivative *II* was also obtained as the sole product of biotransformation of the studied substrate *I* by means of *B. bassiana*. In contrast to this, different results were observed during the hydroxylation of substance *I* by two strains of *R. nigricans* of different origin. The collection strain of *R. nigricans* (Institute of Microbiology, Czechoslovak Academy of Sciences) gave a monohydroxylated compound exclusively, which was identified as the 7β-hydroxy derivative *VII*, while the biotransformation of compound *I* by a freshly isolated *R. nigricans* strain gave a mixture of the same 7β-hydroxyderivative *VII* (46%) with a smaller amount of the 6β-hydroxy compound *X* (10·5%).

From the above results it follows that an unambiguous conclusion concerning the influence of the 21-hydroxy group in compound *I* on the course of its enzymatic transformation by various microorganisms cannot be drawn. From the comparison of the predominant 11α-hydroxylation of substance *I* and 17α-hydroxy-16-methylene-4-pregnene-3,20-dione[9] it follows that the specificity of the hydroxylating system in *B. bassiana* is low. However, the substitution of the 21 position by a hydroxyl function seems decisive for a completely different course of biotransformation of substance *I* by two *R. nigricans* strains of different origin. The more specific hydroxylating system of this classical 11α-hydroxylator represented an obstacle to its potential application in the synthesis of the important intermediate *II*.

EXPERIMENTAL

Beauveria bassiana was obtained from the collection of the Botanical Institute, Biological Faculty, Charles University, Prague. One of the *Rhizopus nigricans* strains originates from the collection of the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, while the other was isolated in our Institute from infected tropical fruits.

Melting points were determined on a Kofler block. Optical rotations were measured in chloroform unless stated otherwise. Experimental error ±4°C. Samples for analysis were dried over phosphorus pentoxide at 0·1 Torr and 100°C for 8 hours. Ultraviolet spectra were measured on a Zeiss, Model VSU spectrophotometer (in methanol), infrared spectra were measured on a double-beam Zeiss spectrophotometer, model UR-10, in chloroform, unless stated otherwise. Chromatography was carried out on thin layers of silica gel bound with calcium sulfate, using chloroform or its mixtures with ethanol as the running phase.

16-Methylene-11α,17α,21-trihydroxy-4-pregnene-3,20-dione (*II*)

Ten litres of a sterile nutritive medium containing 1% of glucose, 1·8% of corn-steep (50% of dry residue), and 0·2% of magnesium sulfate, were brought to pH 6·0 in a 20 l fermentation tank and then inoculated with 200 ml of a suspension of 96 hours old spores inoculum of *B. bassiana*, grown under aerobic conditions on a cultivation medium of the same composition for 22 hours

(aeration 2·5 l/min, stirring 350 rpm, 28°C). The mixture was additioned with a solution of 17α,21-dihydroxy-16-methylene-4-pregnene-3,20-dione (*I*) (3 g) in a mixture of dimethylformamide (20 ml) and ethanol (20 ml) and allowed to be transformed under the same conditions until the starting steroid disappeared (approx. 12 hours). The mycelium was filtered off, washed with dichloromethane, and the combined filtrates were extracted four times with the same solvent (totally 15 l). The organic extract was washed with water, evaporated to dryness to 0·5 l volume, dried over sodium sulfate, and the solvent was evaporated under reduced pressure to dryness. The oily residue was freed from fatty impurities by washing (decantation) with warm light petroleum. The residual solvent was distilled off *in vacuo* and the amorphous substance obtained was crystallised from ethyl acetate. Yield, 1·18 g of 11α-hydroxy derivative *II*, m.p. 202—205°C. The mother liquors gave another crop of the same substance (0·39 g), m.p. 199 to 203°C. Total yield, 1·57 g (50%). Further crystallisation from ethyl acetate gave an analytically pure product, m.p. 206—209°C; $[\alpha]_D^{25}$ +39° (dioxan, *c* 1·3); UV spectrum: λ_{max} 242 nm (log ε 4·19); IR spectrum: 3520, 3430 (OH), 1715 (20-CO), 1650, 1630 (conjugated CO), 870 cm^{-1} (exomethylene). Literature[7] gives m.p. 199—201°C; $[\alpha]_D^{23}$ +42° (dioxan); UV spectrum (methanol) 241 nm (log ε 4·22). For $C_{22}H_{30}O_5$ (374·5) calculated: 70·56 C, 8·08% H; found: 70·31% C, 8·22% H.

11α,21-Diacetoxy-17α-hydroxy-16-methylene-4-pregnene-3,20-dione (*III*)

11α-Hydroxy derivative *II* (20 mg) was acetylated with acetic anhydride (0·05 ml) in pyridine (0·2 ml) at room temperature overnight. The reaction mixture was diluted with icy water and the oily product was extracted with ether. The extract was washed with water, dilute hydrochloric acid, water, potassium hydrogen carbonate solution, and water. It was then dried over sodium sulfate, filtered and the solvent evaporated to dryness. The product, diacetate *III*, would not crystallise, but it was chromatographically pure. Yield, 22 mg; $[\alpha]_D^{22}$ +40·4° (*c* 2·2); UV spectrum: λ_{max} 240 nm (log ε 4·16): IR spectrum: 3580, 3440, (OH), 1055 (C—OH), 1740, 1255, 1030 (CH_3COO), 1725 (20-CO), 1660, 1612 cm^{-1} (conjugated CO). An oily product having identical IR spectrum was obtained on acetylation of the 21-monoacetate *IV* under the same conditions.

21-Acetoxy-11α,17α-dihydroxy-16-methylene-4-pregnene-3,20-dione (*IV*)

Into a solution of 11α-hydroxy derivative *II* (2 g) in pyridine (10 ml), cooled to −20°C, acetic anhydride (0·58 ml) was added and the reaction mixture was allowed to stand at −20°C for 24 hours. The mixture was poured into ice-cold water and the product was extracted with ether which was then worked up in the conventional manner. Crystallisation of the crude product from acetone–heptane gave 1·58 g (71%) of 21-monoacetate *IV*, m.p. 208—212°C. The analytically pure product had m.p. 214—216°C; $[\alpha]_D^{22}$ +38° (*c* 0·9); UV spectrum: λ_{max} 242 nm (log ε 4·22); IR spectrum: 3590, 3430 (OH), 1740 (CH_3COO), 1725 (20-CO), 1660, 1612 cm^{-1} (conjugated CO). For $C_{24}H_{32}O_6$ (416·5) calculated: 69·21% C, 7·74% H; found: 68·90% C, 7·62% H.

21-Acetoxy-17α-hydroxy-16-methylene-4-pregnene-3,11,20-trione (*V*)

Jones reagent (0·23 ml) was added dropwise and under stirring to an ice-cooled solution of 21-monoacetate *IV* (296 mg) in acetone (15 ml). After one hour standing the mixture was treated by the addition of 0·5 ml of 2-propanol in order to decompose excess oxidant, and then concentrated *in vacuo* almost to dryness. The residue was diluted with water and the separated product was filtered off, washed with water, and dried. The filtrate was extracted with ether and the extract was washed with water, dried, and evaporated to dryness. Both fractions were combined and

crystallised from methanol. Yield, 196 mg (66%) of 11-keto derivative *V*, m.p., 219—221°C; $[\alpha]_D^{23}$ +148° (dioxan, *c* 1·1); UV spectrum: λ_{max} 237 nm (log ε 4·22); IR spectrum: 3590, 3440 (OH), 1740, 1260 (21-CH_3COO), 1720 (20-CO), 1710 (six-membered ring CO), 1665, 1618 (conjugated CO), 890 cm^{-1} (exomethylene). Literature[11] gives m.p. 213—214°C, $[\alpha]_D^{23}$ +140° (dioxan), UV spectrum: λ_{max} 237 nm (log ε 4·23). For $C_{24}H_{30}O_6$ (414·5) calculated: 69·54% C, 7·30% H; found: 69·29% C, 7·20% H.

21-Acetoxy-17α-hydroxy-16-methylene-4,9(11)-pregnadiene-3,20-dione (*VI*)

To a solution of 21-monoacetate *IV* (18 mg) in chloroform (0·5 ml) and pyridine (0·05 ml) methanesulfonyl chloride (0·02 ml) was added and the reaction mixture was allowed to stand overnight at room temperature. It was then diluted with water and the product was extracted with ether. The extract was washed with water, dried over anhydrous sodium sulfate and the solvent was distilled off under reduced pressure. The residue was dissolved in acetic acid (1·5 ml) and additioned with freshly remelted sodium acetate (50 mg). The mixture was refluxed for 35 minutes under exclusion of air humidity, then diluted with water, and the product was extracted with ether. The extract was further worked up in the usual manner. The crude product gave on crystallisation from methanol 9 mg (52%) of dehydro compound *VI*, m.p. 217—218°C; $[\alpha]_D^{23}$ +48° (*c* 0·9); according to IR spectra the product was identical with an authentic sample. Literature[11] gives m.p. 215—217°C, $[\alpha]_D$ +51°.

16-Methylene-7β,17α,21-trihydroxy-4-pregnene-3,20-dione (*VII*)

In a two-litre fermentation tank 1 000 ml of the cultivation medium were sterilised. The composition of the medium was the following: glucose 2%, corn-steep liquor (50% dry residue) 3%, potassium dihydrogen phosphate 0·02%, crystalline magnesium sulfate 0·05%; pH before sterilisation 5·0. When the medium was cooled to 28°C it was inoculated with 10 ml of suspension of *R. nigricans* spores (Institute of Microbiology, Czechoslovak Academy of Sciences) and cultivation was carried out at the same temperature and under stirring and aeration for 24 hours. Then a solution of compound *I* (300 mg) in dimethylformamide (3 ml) was added to the mixture and biotransformation was allowed to proceed for 22 hours. The mycelium was filtered off, washed with dichloromethane, and the combined filtrates were extracted with dichloromethane. The crude product was crystallised from ethyl acetate. The first crop of crystals weighed 115 mg, m.p. 206—209°C, the second crop 75 mg, m.p. 205—209°C, *i.e.* the total yield was 190 mg or 61% of 7β-hydroxy derivative *VII*. Further crystallisation increased the m.p. to 211—214°; $[\alpha]_D^{22}$ +26° (dioxan, *c* 1·1); UV spectrum: λ_{max} 242 nm (log ε 4·18); IR spectrum in Nujol: 3480 (OH), 1712 (20-CO), 1640, 1610, 866 (conjugated CO), 1088, 1055 (C-OH), 890 cm^{-1} (exomethylene). For $C_{22}H_{30}O_5$ (374·5) calculated: 70·56% C, 8·08% H; found: 70·44% C, 8·02% H.

7β,21-Diacetoxy-17α-hydroxy-16-methylene-4-pregnene-3,20-dione (*VIII*)

7β-Hydroxy derivative *VII* (40 mg) was acetylated with acetic anhydride (0·2 ml) in pyridine (0·8 ml). The reaction mixture was allowed to stand overnight at room temperature and then worked up in the usual manner. Crystallisation of the crude product from methanol gave 36 mg (74%) of diacetate *VIII*, m.p. 214—215°C; $[\alpha]_D^{23}$ +32° (*c* 3·4); UV spectrum: λ_{max} 238 nm (log ε 4·21); IR spectrum: 3590, 3450 (OH), 1050 (C—OH), 1740, 1255, 1032 (CH_3COO), 1725 (20-CO), 1665, 1620 cm^{-1} (conjugated CO). For $C_{26}H_{34}O_7$ (458·5) calculated: 68·10% C, 7·47% H; found: 68·20% C, 7·41% H.

21-Acetoxy-17α-hydroxy-16-methylene-4,6-pregnadiene-3,20-dione (*IX*)

Diacetate *VIII* (25 mg) was refluxed for 5 hours with acetic acid (2 ml). The mixture was diluted with water and allowed to stand for 48 hours. The separated product was filtered under suction, washed, dried (13 mg, 60%), and crystallised from a mixture of acetone and heptane, m.p. 215 to 216°C; $[\alpha]_D^{22}$ −31° (*c* 0·8); UV spectrum: λ_{max} 285 nm (log ε 4·42); IR spectrum: 3600, 3480 (OH), 1740, 1270 (CH_3COO), 1728 (20-CO), 1658, 1620, 1585 (conjugated CO), 885 cm^{-1} (exomethylene). For $C_{24}H_{30}O_5$ (398·5) calculated: 72·33% C, 7·59% H; found: 72·01% C, 7·44% H.

16-Methylene-6β,17α,21-trihydroxy-4-pregnene-3,20-dione (*X*)

In a three-litre fermentation tank 1500 ml of a nutrition medium of the composition as in the preparation of *VII* was introduced and sterilized. After cooling to 28°C, 10 ml of a suspension of spores of a freshly isolated strain of *R. nigricans* were added, and the mixture was cultivated at 28°C for 20 hours under aeration and stirring. To the obtained suspension of the mycelium a solution of compound *I* (450 mg) in dimethylformamide (4 ml) was added and the biotransformation was allowed to proceed for 20 hours. The mycelium was then filtered off, the filtrate was extracted with dichloromethane, the latter was evaporated to dryness and the residue was washed with light petroleum by decantation. The crude product weighed 196 mg and it was chromatographed on a silica gel column (20 g). Elution with chloroform–benzene (2 : 1) gave 40 mg of the starting compound *I*, elution with chloroform alone gave 6β-hydroxy derivative *X* (49 mg, 10·5%), m.p. 256–260°C (ethyl acetate); $[\alpha]_D^{20}$ −34° (*c* 0·5); UV spectrum: λ_{max} 234 nm (log ε 4·20); IR spectrum: 3580, 3470 (OH), 1715 (20-CO), 1630, 1610 (conjugated CO), 885 cm^{-1} (exomethylene). For $C_{22}H_{30}O_5$ (374·5) calculated: 70·56% C, 8·08% H; found: 70·31% C, 7·91% H. Elution with chloroform–ethanol (99·1) gave 220 mg (47%) of 7β-hydroxy derivative *VII*, m.p. 208–210°C, identical with the product described above.

6β,21-Diacetoxy-17α-hydroxy-16-methylene-4-pregnene-3,20-dione (*XI*)

6β-Hydroxyderivative *X* (22 mg) was acetylated with acetic anhydride (0·05 ml) in pyridine (0·1 ml) at room temperature overnight. It was worked up in the conventional manner and the crystalline residue was crystallised from methanol to give 18 mg (67%) of diacetate *XI*, m.p. 201–202°C; $[\alpha]_D^{22}$ −5° (*c* 2·0); UV spectrum: λ_{max} 235 nm (log ε 4·10); IR spectrum: 3600, 3480 (OH), 1730 (CH_3COO), 1675, 1620 (conjugated CO), 891 cm^{-1} (exomethylene). For $C_{26}H_{34}O_7$ (458·5) calculated: 68·10% C, 7·47% H; found: 67·79% C, 7·30% H.

Reaction with zinc in acetic acid: Diacetate *XI* (6 mg) was shaken with zinc powder (10 mg) in glacial acetic acid (0·1 ml) overnight. The solvent was distilled off *in vacuo* and the dry residue was diluted with water and the product extracted with ether. The extract was worked up in the usual manner to give a crystalline product of m.p. 157–159°C the infrared spectrum of which was identical with that of authentic 21-acetoxy-17α-hydroxy-16-methylene-4-pregnene-3,20-dione (*XII*) (ref.[11]).

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