STEROID DERIVATIVES. LXXIII.*

HYDROXYLATION OF SUBSTANCES OF PREGNANE SERIES WITH *Coniosporium rhizophilum* (PREUSS)

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Microbial hydroxylation of Reichstein's substance S with *Coniosporium rhizophilum* (PREUSS) gave a mixture of 1β-hydroxy derivative *II* and epicortisol *III*, while progesterone and deoxycorticosterone acetate afforded under the same conditions corresponding 1β,6β-dihydroxy derivative *VI* or *XIV*, respectively. In the latter case the 21-acetoxy group underwent hydrolysis simultaneously.

In connection with the study of hydroxylation of pregnane derivatives with various microorganisms we also investigated the structure of products formed under the influence of *Coniosporium rhizophilum* (PREUSS) on various steroid substrates. The biotransformation of steroid compounds with this microorganism has not been studied as yet.

On hydroxylation of 17α,21-dihydroxy-4-pregnene-3,20-dione (Reichstein's substance S, *I*) with *C. rhizophilum* a mixture of compounds was obtained in which two products predominated. According to their lowered R_F values on thin layers they were evidently the products of monohydroxylation of the starting compound. Both products were separated chromatographically on silica gel. Less polar compound *II*, isolated in a 16% yield, gave on dehydration with acetic acid at elevated temperature the known 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (*IV*) (ref.[1]) which indicated the presence of β-hydroxyketone grouping in the molecule. The physical constants of substance *II* were identical with those of 1β,17α,21-trihydroxy-4-pregnene-3,20-dione[2,3] and its structure was demonstrated by comparison with an authentic sample. Chromatography on silica gel further gave a more polar product in 11% yield, to which the structure of 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (epicortisol, *III*) was assigned on the basis of its physical constants and comparison with an authentic sample.

1-Hydroxylation of pregnene type compounds is not particularly common[4], and in all instances it gives low yields. It can be performed by *Rhizoctonia ferrugena*[2], *Streptomyces*[5], *Mortierella*[6], *Cladosporium*[7], and *Absidia orchidis*[8].

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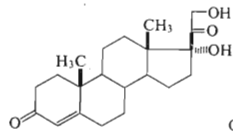
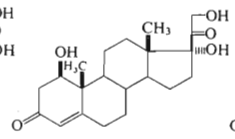
A more complex hydroxylation pattern was observed with 4-pregnene-3,20-dione (*V*). The crude reaction product was composed according to thin-layer chromatography of a main component – according to a distinct decrease of the R_F value evidently a dihydroxylated progesterone – accompanied by several additional substances of similar polarity. Chromatography of this mixture on silica gel gave main component, substance *VI*, which according to elemental analysis contained two hydroxyl functions and the ultraviolet spectrum of which showed an appreciable hypsochromic shift of the absorption maximum (235 nm). The UV maximum suggested the presence of a hydroxy group in position 6β (ref.[9]). The dihydroxy compound *VI* was acetylated in pyridine with acetic anhydride at room temperature to give corresponding diacetate *VII*. The smooth dehydration of compound *VI* in hot acetic acid indicated that one of the hydroxy groups must be in the β-position to the carbonyl, *i.e.* in position 1. The product *IX* obtained on dehydration was formulated as a 1,4-pregnadien-3-one, which was corroborated by its ultraviolet spectrum. Acetylation of *IX* under mild conditions gave monoacetate *X* which on alkaline hydrolysis gave back the starting alcohol *IX*. This reaction also excluded the presence of the acetoxy group in position 7. In contrast to this, when an attempt was made to esterify the monohydroxy compound *IX* with *p*-toluenesulfonyl chloride in pyridine the starting material was regenerated, which demonstrates the impaired accessibility of the hydroxyl function.

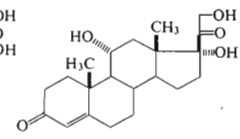
The assumption that dihydroxy compound *VI* has the hydroxy groups in positions 1 and 6β was proved by NMR spectroscopy. In view of the limited solubility of diol *VI*



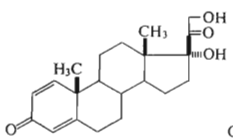
I



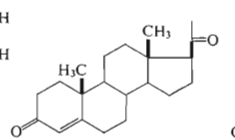
II



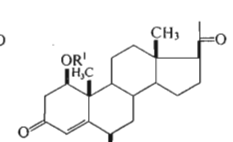
III



IV



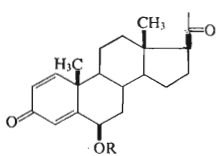
V



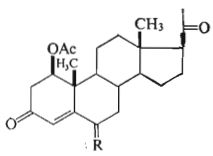
VI, $R^1 = R^2 = H$
VII, $R^1 = R^2 = Ac$
VIII, $R^1 = Ac$, $R^2 = H$

in deuteriochloroform, the spectra of diacetate *VII* and 1-dehydro compound *IX* and *X* were measured instead. The protons of compound *VII*, bound to the carbon carrying the ester function, showed signals corresponding to signals of protons $H_{1\alpha}$ and $H_{6\alpha}$ in hydroxylated Δ^4-3-ketosteroids[10]. In the spectrum of diacetate *VII* a distinct doublet of doublets was present (δ 5·17 and 5·27) and a triplet (δ 5·48), while 1-dehydro derivatives *IX* and *X* gave only a triplet (δ 5·58 or 5·48). In view of the different dihedral angle formed by the axial 1α-proton with the axial 2β- and equatorial 2α-proton the spectrum of compound *VII* contains a doublet of doublets, in contrast to the equatorial 1β-proton which forms with both protons in position 2 a dihedral angle of almost the same magnitude and which is therefore reflected in the spectrum as a triplet[10]. The same situation occurs in the case of the equatorial 6α-proton which forms an almost identical dihedral angle with both protons in position 7 and also gives a triplet, as was observed in the spectra of substances *IX* and *X*. The *J* values found are in good accordance with the values calculated on the basis of the measurements of angles between single protons, carried out with the aid of Dreiding models[11]. Hence, from the PMR spectra it follows that substance *VI* has unambiguously the structure of 1β,6β-dihydroxyprogesterone.

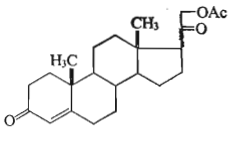
The results of the attempt at partial acetylation of the equatorial 1β-hydroxy group of diol *VI*, are in agreement with this conclusion. By acetylation of this compound with acetic anhydride in pyridine at room temperature (2 hours) a mixture was obtained which according to thin-layer chromatography contained in addition to the unreacted substance *VI* (R_F 0·09) and diacetate *VII* (R_F 0·54) also substance *VIII* of R_F 0·25. Substance *VIII* was isolated by preparative thin-layer



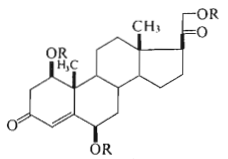
IX, R = H
X, R = Ac



XI, R = O
XII, R = H_2



XIII



XIV, R = H
XV, R = Ac

chromatography and it was formulated as 1β-acetoxy-6β-hydroxy-4-pregnene-3,20-dione (*VIII*). On oxidation with Jones reagent[12] 6β-hydroxy derivative *VIII* was transformed to 1β-acetoxy-4-pregnene-3,6,20-trione (*XI*) with a characteristic UV maximum (253 nm)[13]. The smooth reductive elimination of the 6β-acetoxy group of diacetate *VII* with zinc in acetic acid[14] which gave rise to the known 1β-acetoxyprogesterone (*XII*) (ref.[3]), is in full agreement with the proposed structure.

21-Acetoxy-4-pregnene-3,20-dione (*XIII*) was transformed by *C. rhizophilum* to a mixture from which a product of the triol type was isolated by chromatography on silica gel. In this case too dihydroxylation took place with simultaneous hydrolysis of the 21-acetoxy group. The product formed displayed a distinct hypsochromic shift of its UV maximum at 236 nm, and the differences of molecular rotations of triol *XIV* and its triacetate *XV* with respect to 21-hydroxy-4-pregnene-3,20-dione or its 21-acetate *XIII* corresponded well to the differences in molecular rotation of the dihydroxy derivative *VI* and its diacetate with respect to progesterone (*V*) (Table I). These facts and the smooth dehydration of trihydroxy derivative *XIV* to 1-dehydro compound under mild conditions indicated that the product is again a 1β,6β-dihydroxy derivative. This conclusion was corroborated by the NMR spectrum of triacetate *XV*, in which again a doublet of doublets (δ 5·08 and 5·23) and a triplet (δ 5·45) having an analogous shape to the signals $H_{1\alpha}$ and $H_{6\alpha}$ of the compound *VII* were clearly evident. Therefore, substance *XIV* may be formulated as 1β,6β,21-trihydroxy-4-pregnene-3,20-dione.

After the determination of the structure of the main products of biotransformation we concluded that the enzymatic system of *C. rhizophilum* has an as yet unknown ability to introduce the hydroxy groups simultaneously into position 1β and 6β of pregnane compounds. Microbial dihydroxylation into positions 1 and 6 was described earlier only in the case of two microorganisms, a *Xylaria* sp. (*Ascomycetes, Sphaeriales*)[15] with which 1β,6β-dihydroxy-4-androstene-3,17-dione was prepared from the starting deoxy derivative, and a *Penicillium* sp. (*Fungi imperfecti, Moniliales*)[16], which as a by-product of testosterone hydroxylation was obtained in a 0·2%

TABLE I
Molecular Rotation Differences

Starting compound	$[\Phi]_D$	Product	$[\Phi]_D$	$\Delta[\Phi]_D$
V[a]	+555°	*VI*[a]	+256°	299°
Deoxycorticosterone[a]	+560°	*XIV*[a]	+246°	314°
V[b]	+630°	*VII*[b]	+135°	495°
XIII[b]	+644°	*XV*[b]	+166°	478°

[a] In dioxane, [b] in chloroform.

yield, having the structure of 1α,6β-dihydroxytestosterone. However, in both mentioned microorganisms the simultaneous introduction of hydroxy groups into positions 1 and 6 is limited to androstane derivatives.

EXPERIMENTAL

Microorganism. Coniosporium rhizophilum (PREUSS) is a saprophitic fungus from the class *Fungi imperfecti*, order *Moniliales*, family *Dematiaceae*, *genus Coniosporium* (synonym *Papularia*). It was isolated in our laboratory as a contamining micro-organism from a culture of *Septomyxa affinis* No 2416 obtained in 1962 from the CBS collection (Barn, Holland). It is kept by monthly transfer on to fresh malt agar. Well sporulated cultures are kept at +4°C.

Methods: Melting points (uncorrected) were measured on a Kofler microblock. Optical rotations were measured in chloroform, unless stated otherwise, with a ±3° precision. Samples for analysis were dried over phosphorus pentoxide at 0·1 Torr and 76°C for 8 hours. The UV spectra were measured on a Zeiss, model VSU-1 spectrophotometer (with NaCl prisms and a quartz cell 1 cm thick), in methanol. The IR spectra were taken on a double-beam spectrophotometer Zeiss, model UR-10, in 6% chloroform solutions, unless stated otherwise. The NMR spectra were measured on a Varian HA-100 apparatus at 100 MHz in deuteriochloroform, using tetramethylsilane as the internal standard, with the exception of substance *XIV* which was measured on a Zeiss ZKR 60 machine at 60 MHz. The values of chemical shifts are expressed in p.p.m. of a δ-scale. Thin-layer chromatography was carried out on silica gel CH (Lachema, Brno), in chloroform–methanol mixtures of various compositions. Detection was carried out with concentrated sulfuric acid and subsequent heating at 110°C. Preparative thin-layer chromatography was carried out on silica gel (Kieselgel GF_{254} Merck Darmstadt), using a 1 mm thick layer and applying approximately 2 mg/1 cm of substance.

Microbial Hydroxylation of Reichstein's Substance S (*I*)

Hydroxylation was carried out in a 20 l fermentation tank provided with aeration and stirring (2 500 r.p.m.), filled with 10 l of fermentation medium of the following composition: 3% of glucose, 2% of corn-steep extract (60% of dry weight), 0·2% of potassium hydrogen phosphate, and 0·05% of magnesium sulfate. The pH of the medium before sterilisation was 6·3. The sterile medium in the fermentation tank was inoculated with 5% of a 72 hours old vegetative inoculum grown on a nutrient medium of the same composition. The cultivation was carried out for 24 hours under aeration (3 lt/min) and stirring at 25°C. Then a solution of substance *I* (3 g) in dimethylformamide (25 ml) and ethanol (25) was added to the medium. Transformation was allowed to continue until the starting material disappeared completely, which lasted about 16 hours. The mycelium was filtered off through a loosely-woven tissue, pressed out, washed with water, and the washings were combined with the filtrate. The combined solutions were extracted three times with butyl acetate (15 l *in toto*). The organic extract was washed with a 2% solution of sodium hydrogen carbonate and water, and concentrated *in vacuo* at 50°C (bath temperature). The crude product was decanted with hot light petroleum and chromatographed on a silica gel column (120 g). On elution with a mixture of chloroform and methanol (95 : 5) a chromatographically pure substance (775 mg) was obtained which after double crystallisation from ethyl acetate gave 495 mg (16%) of 1β,17α,21-trihydroxy-4-pregnene-3,20-dione (*II*), m.p. 202—205°C; $[\alpha]_D^{20}$ +86° (dioxane, *c* 2·1); UV spectrum: λ_{max} 241 nm (log ε 4·18). Lit.[2,3] gives m.p. 203—207°C: $[\alpha]_D$ +89° (dioxane). On heating in acetic acid at 100°C for 6 hours, substance *II* was transformed

quantitatively to 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (*IV*), m.p. 245–247°C; $[\alpha]_D^{24}$ +78° (*c* 1·4); UV spectrum: λ_{max} 244 nm (log ε 4·20). Lit.[1] gives m.p. 246–249°C; $[\alpha]_D$ +76°; UV spectrum: λ_{max} 244 nm (log ε 4·21). Elution with a chloroform–methanol mixture (94 : 6) gave a product which on crystallisation from ethyl acetate gave 343 mg (11%) of 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (*III*), m.p. 208–210°C; $[\alpha]_D^{19}$ +116° (methanol, *c* 2·3); UV spectrum: λ_{max} 242 nm (log ε 4·17). Lit.[17,18] gives m.p. 209–212°C; $[\alpha]_D$ +113° (methanol); UV spectrum: λ_{max} 242 nm (log ε 4·17).

Microbial Hydroxylation of 4-Pregnene-3,20-dione (*V*)

Microbial hydroxylation of progesterone (*V*) (3 g), carried out under the conditions given for Reichstein's substance S, gave a crude product which was chromatographed on 120 g of silica gel. Elution of the column with chloroform–methanol (98 : 2) afforded 349 mg of a substance which was crystallised twice from ethyl acetate. Yield: 283 mg (9%) of 1β,6β-dihydroxy-4-pregnene-3,20-dione (*VI*), m.p. 234–236°C; $[\alpha]_D^{19}$ +74° dioxane, *c* 0·8); UV spectrum: λ_{max} 235 nm (log ε 4·16); IR spectrum: 3 420 (hydroxyl), 1 690 ($C_{(20)}$-ketone), 1 356 (methyl ketone), 1 660, 1 610 cm^{-1} (conjugated ketone). For $H_{21}H_{30}O_4$ (346·5) calculated: 72·80% C, 8·73% H; found 72·57% C, 8·69% H.

1β,6β-Diacetoxy-4-pregnene-3,20-dione (*VII*)

Compound *VI* (105 mg) was dissolved in pyridine (0·6 ml) and acetic anhydride (0·3 ml) and allowed to stand at room temperature overnight. The mixture was poured into icy water and the precipitated product was filtered off, washed with water, dried and crystallised from methanol. Pure diacetate *VII* (90 mg, 69%) was obtained, m.p. 147–149°C; $[\alpha]_D^{21}$ +31·4° (*c* 0·9); UV spectrum: λ_{max} 236 nm (log ε 4·18); IR spectrum: 1 740, 1 240, 1 030 (acetate), 1 710 ($C_{(20)}$-ketone), 1 356 (methyl ketone), 1 655, 1 615 cm^{-1} (conjugated ketone); NMR spectrum: 0·70 (18-H), 1·34 (19-H), 2·07 (acetate-H), 2·12 (21-H), 5·17, 5·27 (dd, $J_{1,2\beta}$ = 10 Hz, $J_{1,2\alpha}$ = 4 Hz, 1α-H), 5·48 (t, J = 4 Hz, 6α-H), 6·08 (4-H). For $C_{25}H_{34}O_6$ (430·5) calculated: 69·74% C, 7·96% H; found: 69·77% C, 8·27% H.

6β-Hydroxy-1,4-pregnadiene-3,20-dione (*IX*)

A mixture of substance *VI* (150 mg) and glacial acetic acid (3 ml) was heated at 100°C for 3 hours, diluted with water, and the separated product was filtered off, washed with water, dried, and crystallised from methanol. Yield 105 mg (74%) of 1-dehydro compound *IX*, m.p. 229–231°C; $[\alpha]_D^{19}$ +91° (*c* 2·1); UV spectrum: λ_{max} 246 nm (log ε 4·23); IR spectrum: 3 390 (hydroxyl), 1 700 ($C_{(20)}$-ketone), 1 356 (methyl ketone), 1 658, 1 610, 910 cm^{-1} (conjugated ketone); NMR spectrum: 0·73 (18-H), 1·43 (19-H), 2·11 (21-H), 4·58 (t, *J* = 4 Hz, 6α-H), 6·21 (2,4-coupling d, $J_{1,2}$ = 10 Hz, $J_{2,4}$ = 2 Hz, 2-H), 6·15 (4-H), 7·06 (d, *J* = 10 Hz, 1-H). For $C_{21}H_{28}O_3$ (328·4) calculated: 76·79% C, 8·59% H; found: 76·39% C, 8·61% H.

6β-Acetoxy-1,4-pregnadiene-3,20-dione (*X*)

A mixture of 6β-hydroxy derivative *IX* (90 mg), acetic anhydride (0·15 ml), and pyridine (0·6 ml) was allowed to stand at room temperature overnight. It was then diluted with ice-water and the separated product was extracted with ether. The extract was washed with water, dilute hydrochloric acid, water, sodium hydrogen carbonate solution, and again with water, and then dried over sodium sulfate and filtered. The solvent was distilled off *in vacuo* to dryness. Crystallisation of the residue from methanol gave acetate *X* (75 mg, 74%), m.p. 151–152°C; $[\alpha]_D^{19}$ +54·5°

(*c* 0·4); UV spectrum: λ_{max} 244 nm (log ε 4·16); IR spectrum: 1735, 1240, 1024 (acetate), 1700 ($C_{(20)}$-ketone), 1356 (methyl ketone), 1663, 1625, 960 cm^{-1} (conjugated ketone), hydroxyl absent; NMR spectrum: 0·72 (18-H), 1·30 (19-H), 2·03 (acetate-H), 2·09 (21-H), 5·48 (t, J = 3 Hz, 6α-H), 6·21 (2,4-coupl. d, $J_{1,2}$ = 10 Hz, $J_{2,4}$ = 2 Hz, 2-H), 6,28 (4-H), 7·02 (d, J = 10 Hz, 1-H). For $C_{23}H_{30}O_4$ (370·5) calculated: 74·56% C, 8·16% H; found: 74·61% C, 8·04% H.

1β-Acetoxy-6β-hydroxy-4-pregnene-3,20-dione (*VIII*)

A mixture of substance *VI* (100 mg), acetic anhydride (0·3 ml), and pyridine (0·6) was allowed to stand at room temperature for 2 hours and then poured into cold water. The precipitate was extracted with ether and the organic extract was worked up in the conventional manner. The crude product was separated by preparative thin-layer chromatography in chloroform–methanol (99 : 1). From the more polar zone 21 mg of the starting diol *VI* were recovered, while from the least polar zone 28 mg of diacetate *VII* were isolated. The zone of medium polarity gave after isolation 35 mg of a product which was crystallised from methanol. Yield 24 mg (21%) of monoacetate *VIII*, m.p. 102—104°C; $[\alpha]_D^{24}$ +66° (*c* 1·0); UV spectrum: λ_{max} 236 nm (log ε 4·16); IR spectrum: 3400 (hydroxyl), 1745, 1240, 1030 (acetate), 1700 ($C_{(20)}$-ketone), 1356 (methyl ketone), 1670, 1620 cm^{-1} (conjugated ketone). For $C_{23}H_{32}O_5$ (388·5) calculated: 71·10% C, 8·30% H; found: 70·91% C, 8·35% H.

1β-Acetoxy-4-pregnene-3,6,20-trione (*XI*)

Monoacetate *VIII* (12 mg) in acetone (1 ml) was oxidised with excess Jones reagent for 1 minute. The excess was decomposed with a drop of methanol, the mixture was diluted with water, the product was extracted with ether, and the organic layer was worked up in the usual manner. A product (7 mg), chromatographically almost pure, was isolated which would not crystallise. UV spectrum: λ_{max} 253 nm (log ε 3·90), IR spectrum without maximum for the hydroxy group. The ultraviolet maximum[13] corroborates the proposed structure.

1β-Acetoxy-4-pregnene-3,20-dione (*XII*)

1β,6β-Diacetate *VII* (50 mg) was shaken with zinc dust (200 mg) in glacial acetic acid (1 ml) at room temperature for 8 hours. Zinc was filtered off and washed with a small amount of acetic acid. The filtrate was diluted with water and the product extracted with ether. The organic layer was washed with a solution of potassium hydrogen carbonate and water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. Crystallisation of the residue from methanol gave 24 mg (56%) of 1β-acetoxy derivative of progesterone (*XII*), m.p. 171—174°C; $[\alpha]_D^{24}$ +68° (*c* 1·4). Literature[3] gives m.p. 174—176°C; $[\alpha]_D$ +71°.

Microbial Hydroxylation of 21-Acetoxy-4-pregnene-3,20-dione (*XIII*)

21-Acetoxy-4-pregnene-3,20-dione (*XIII*) (3 g) was hydroxylated in the same manner as described for the hydroxylation of compound *I*. The crude product (2·6 g) was chromatographed on a silica gel column (130 g). Elution with chloroform–methanol (92 : 8) gave 520 mg of substance which was crystallised thrice from ethyl acetate. Yield 282 mg (10%) of 1β,6β,21-trihydroxy-4-pregnene-3,20-dione (*XIV*), m.p. 198—199°C; $[\alpha]_D^{23}$ +68° (dioxane, *c* 1·1); UV spectrum: λ_{max} 236 nm (log ε 4·17); IR spectrum (nujol): 3350 (hydroxyl), 1718 ($C_{(20)}$-ketone), 1668, 1610 (conjugated ketone), 1076, 1039 cm^{-1} (C—O—H), acetate absent. For $C_{21}H_{30}O_5$ (362·5) calculated: 69·58% C, 8·34% H; found: 69·00% C, 8·20% H. The substance was converted in acetic acid at 100°C to a substance of λ_{max} 244 nm (log ε 4·18) after 6 hours heating.

1β,6β,21-Triacetoxy-4-pregnene-3,20-dione (*XV*)

A mixture of trihydroxy derivative *XIV* (100 mg), acetic anhydride (0·4 ml) and pyridine (0·8 ml) was allowed to stand at room temperature overnight. The reaction mixture was poured onto ice and worked up in the conventional manner. Crystallisation of the crude product from methanol gave 75 mg (55%) of triacetate *XV*, m.p. 186—188°C; $[\alpha]_D^{22}$ +34° (*c* 2·3); UV spectrum: λ_{max} 236 nm (log ε 4·17); NMR spectrum: 0·73 (18-H), 1·34 (19-H), 2·04, 2·13 (acetate-H), 4·58 (d, J = 6 Hz, 21-H), 5·08, 5·23 (dd, $J_{1,2\beta}$ = 10 Hz, $J_{1,2\alpha}$ = 5 Hz, 1α-H), 5·46 (t, J = 3 Hz, 6α-H), 6·03 (4-H). For $C_{27}H_{36}O_8$ (488·6) calculated: 66·37% C, 7·43% H; found: 66·04% C, 7·58% H.

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