MICROBIAL OXYGENATION OF B-NORSTEROIDS WITH *Beauveria bassiana**

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Microbial oxygenation of the B-nor analogue of Reichstein's substance S and of 3β,17α,21-trihydroxy-B-norpregn-5-en-20-one with *Beauveria bassiana* have been studied and the structures of the products isolated were established by spectral as well as chemical means.

In some of our previous papers we dealt with syntheses of B-noranalogues of corticoids. We described[1] synthesis of the B-noranalogue of Reichstein's substance S, studied the conditions of microbial oxygenation of some B-norpregnane derivatives[2] and B-norandrostane derivatives[3] and recently we have described a chemical synthesis of B-norcortisol[4] in 15-step reaction sequence. The low yields of this synthesis necessitated further studies which are presented in this paper.

A possibility which remained to be proved was microbial oxygenation of the B-noranalogue of Reichstein's substance S (*I*). From a number of microorganisms we investigated, only *Beauveria bassiana* proved suitable for our purpose. Oxygenation of the B-norderivative *I* with this microorganism afforded four products which were separated by column chromatography on silica gel. All of them, as detected by mass spectrometry, were products of monohydroxylation. The most lipophilic triol was obtained in 15% yield and removal of the ketol side chain by sodium bismuthate oxidation[5] led to the known[3] androstane derivative *IV*. This product is therefore a product of 6α-hydroxylation and has structure *II*. As was detected by spectral means the mother liquors after crystallisation of triol *II* contained some other component identical in polarity with the triol *II*. After acetylation of these mother liquors this compound was separated by chromatography as the more polar component. It proved to be a monoacetate, newly introduced hydroxyl being left unesterified under normal conditions of acetylation (room temperature, 18 h). This hydroxyl is therefore in 11β-position and comparison with the authentic[4] B-norcortisol acetate proved this presumption. This product was therefore the triol *V* and the acetate has structure *VI*. The third product afforded after oxidative removal

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of the side chain the known[3] 11α-hydroxy derivative *IX*. It is therefore the triol *VII*. The most polar product was the triol *X*. The structure was assigned on the basis of analogy with hydroxylation of the triol *XII*, where the structure of the 15α hydroxylated derivative *XVI* was established by ^{1}H-NMR spectrometry. The main product of this biotransformation was the 6α alcohol *II* and only very small part of the substance underwent 11β hydroxylation.

We therefore carried out further experiments with triol *XII* in which the position 6 is not activated. Three products of monohydroxylation were detected in the reaction mixture. Acetylation of the lipophilic component yielded a diacetate leaving the newly introduced hydroxyl unaffected. This lipophilic product is therefore the ketol *XIII* and the acetate has structure *XIV*. This was proved by transformation to the known[4] bis(methylenedioxy) derivative *XV*. The second product was a B-nortetrol which afforded a triacetate on acetylation. Oxidation with sodium bismuthate yielded a 17-oxoandrostene derivative with a hydroxyl in 15α position as follows from the ^{1}H-NMR spectra: The protons at $C_{(16)}$ appear as doublet of doublets (1·9 and 2·81 ppm) and decoupling experiments proved the interaction with the 15α--proton. The tetrol is therefore the 15α-hydroxy derivative *XVI*, the triacetate has structure *XVII*. The third, most polar product was the 11α-hydroxy derivative *XIX*. This structure was securely established again by oxidative removal of the side chain to the known[3] 11α-hydroxy-B-norandrostane derivative *XXI*.

These experiments have shown that hydroxylation of the B-nortriol *XII* with *Beauveria bassiana* represents a promising pathway for synthesis of B-norcorticoids.

EXPERIMENTAL

Melting points were determined on a Kofler block. Analytical samples were dried at 80°C 0·2 Torr. Optical measurements were carried out in ethanol unless otherwise stated with an error of ±1°. The infrared spectra were recorded on a Zeiss UR 10 spectrometer in tetrachlormethane unless otherwise stated. The mass spectra were recorded on a AEI MS 902 mass spectrometer. The ^{1}H-NMR spectra were recorded on a Varian HA-100 instrument in $CDCl_3$–hexadeuteriodimethyl sulphoxide (1 : 1) with tetramethylsilane as internal reference at 30°C. Chemical shifts are given in ppm. The multiplicity is expressed: s (singlet), d (doublet), m (multiplet), *W* (width of the multiplet). Apparent coupling constants were obtained from the first order analysis. The identity of samples prepared by different routes was checked by mixture melting point determination, by thin layer chromatography (TLC) and by infrared spectra. Usual working up of an ethereal solution implies washing the solution with 5% aqueous hydrochloric acid, water, 5% aqueous potassium hydrogen carbonate solution, water, drying with magnesium sulfate and evaporation of the solvent *in vacuo*. Light petroleum refers to the fraction of b.p. 45–60°C.

6α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione (*II*)

A fermentation medium (1 000 ml) containing 1% of glucose, 1·8% of corn steep oil, 0·2% of cryst. magnesium sulphate was adjusted with 10% potassium hydroxide to a pH of 6·0, sterilised at 120°C for 1 h and inoculated with 100 ml suspension of a 96 h old culture of *Beauveria bassiana*

CH2OR
C=O
OH
HO
O
V, R = H
VI, R = Ac
IV
I
IX
AcO
X, R = H
XI, R = Ac
II R = H
III. R = Ac
OR
VII, R = H
VIII. R = Ac
RO

XVI, R = H
XVII, R = Ac
XV
XII
XVIII
XXI
XIII, R = H
XIV, R = Ac
XIX, R = H
XX, R = Ac

prepared under similar conditions from an agar culture. After 24 h at 28°C the cultivation medium was treated with a solution of the B-noranalogue of Reichstein's substance S (*I*) [250 mg] in dimethylformamide–ethanol (1 : 1) [5 ml]. The biotransformation was continued under the same conditions for about 30 h, when the starting steroid was no more detectable. The mycelium was then filtered off, washed with butyl acetate (3 . 500 ml) and the filtrate was extracted with the same solvent. The combined extracts were washed with a sodium carbonate solution (5%), water, dried with magnesium sulphate and the solvent was distilled off under reduced pressure. The residue was washed with light petroleum (2 . 100 ml) yielding 260 mg of the crude product of biotransformation containing, according to TLC, three components. The mixture was chromatographed on a silica gel column (50 g) in chloroform–methanol (19 : 1). Fractions with the lipophilic component were worked up to yield 35 mg of a crude product which after crystallisation from acetone gave 15 mg of the 6α,17α,21-triol *II*, m.p. 222–225°C, $[\alpha]_D^{20}$ +140° (*c* 1·3). Mass spectrum: M^+ 348. ^{1}H-NMR: 0·60 (s, 18-H), 1·05 (s, 19-H), 5·93 (s, 4-H), 5·08 (s, 17-OH) 4·19 and 4·59 (2 d, J = 19·5 Hz, 21-H), 4·44 (d, 6-H). For $C_{20}H_{28}O_5$ (348·4) calculated: 68·94% C, 8·10% H; found: 68·51% C, 8·11% H.

6α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione 6,21-diacetate (*III*)

The triol *II* (20 mg) was acetylated with acetic anhydride (0·2 ml) in pyridine (0·4 ml) for 18 h at room temperature. The mixture was decomposed with a saturated sodium chloride solution, the product was taken into ethyl acetate and the extract was worked up. The residue was crystallised from acetone to yield 18 mg of the diacetate *III*, m.p. 167–168°C. Mass spectrum: M^+ 432.

6α-Hydroxy-B-norandrost-4-en-3,17-dione (*IV*)

A solution of the dione *II* (6 mg) in 50% acetic acid (0·2 ml) was treated with sodium bismuthate (60 mg) and agitated in darkness for 3 h. The mixture was diluted with water, the product taken into ethyl acetate and the extract was worked up to yield 4 mg of the dione *IV*, m.p. 210°C, identical in all respects with the authentic[3] sample.

11β,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione 21-acetate (*VI*)

The mother liquors obtained after crystallisation of the triol *II* from acetone (20 mg) were acetylated with acetic anhydride (0·2 ml) in pyridine (0·4 ml) overnight at room temperature. Usual working up afforded a product consisting of two components. They were separated by preparative thin layer chromatography in chloroform–methanol (19 : 1). The lipophilic component was the diacetate *III*, the polar compound was crystallised from acetone to yield 1·8 mg of the monoacetate *VI*, m.p. 171–173°C, identical in all respects with the acetate prepared by acetylation of the authentic B-norcortisol *V*. Mass spectrum: M^+ 390.

11α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione (*VII*)

Elution of the chromatography after isolation of the triol *II* with the same solvent mixture (chloroform–methanol 19 : 1) afforded fractions with the third product of hydroxylation (30 mg). Crystallisation from acetone gave 10 mg of the B-nortriol *VII*, m.p. 205–208°C, $[\alpha]_D^{20}$ +160° (*c* 1·2). Mass spectrum: M^+ 348. ^{1}H-NMR: 0·67 (s, 18-H), 1·12 (s, 19-H), 5·74 (s, 4-H), 5·22 (s, 17-OH), 4·28 and 4·61 (2 d, J = 20 Hz, 21-H), 3·95 (m, W = 30 Hz, 11β-H). For $C_{20}H_{28}O_5$ (348·4) calculated: 68·94% C, 8·10% H; found: 68·61% C, 8·12% H.

11α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione 11,21-diacetate (*VIII*)

The triol *VII* (20 mg) in pyridine (0·4 ml) was acetylated with acetic anhydride (0·2 ml) for 18 h at room temperature. The mixture was decomposed with a saturated sodium chloride solution, the product extracted into ethyl acetate and the solution was worked up. The residue (23 mg) after evaporation of the solvent was chromatographed over silica gel (5 g) in benzene–ether (1 : 1). Working up of fractions containing the acetate and crystallisation from ethyl acetate yielded 15 mg of the diacetate *VIII*, m.p. 162–163°C. Mass spectrum: M^+ 432.

11α-Hydroxy-B-norandrost-4-en-3,17-dione 11-acetate (*IX*)

A solution of the diacetate *VIII* (9 mg) in 50% acetic acid (0·3 ml) was treated with sodium bismuthate (120 mg) and agitated in darkness for 6 h at room temperature. The mixture was diluted with ethyl acetate, treated with water and the organic layer was worked up and the residue after evaporation of the solvent (7 mg) was purified by preparative TLC in chloroform–methanol (19 : 1). The products were extracted with ethyl acetate to yield 4·5 mg of the starting diacetate *VIII* and 1·2 mg of the dione *IX*, m.p. 165–166°C, identical in all respects with the authentic[3] compound.

15α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione (*X*)

Elution of the chromatography after isolation of the triols *II* and *VII* with chloroform–methanol (9 : 1) afforded fractions with the most polar product. Working up gave 30 mg of crystalls which on crystallisation from acetone yielded 10 mg of the triol *X*, m.p. 224–226°C, $[\alpha]_D^{20}$ +110° (*c* 1·1). Mass spectrum: M^+ 348. ^{1}H-NMR: 0·69 (s, 18-H), 1·15 (s, 19-H), 5·66 (s, 4-H), 5·19 (s, 17-OH), 4·15 and 4·55 (2 d, J = 20 Hz, 21-H), 3·78 (m, W = 26 Hz, 15β-H). For $C_{20}H_{28}O_5$ (348·4) calculated: 68·94% C, 8·10% H; found: 68·66% C, 8·09% H.

15α,17α,21-Trihydroxy-B-norpregn-4-en-3,20-dione 15,21-diacetate (*XI*)

The triol *X* (20 mg) in pyridine (0·4 ml) was acetylated with acetic anhydride (0·2 ml) at room temperature for 18 h. The excess anhydride was removed with a saturated sodium chloride solution and the product was isolated with ethyl acetate. The solution was worked up and the residue (25 mg) was purified by preparative TLC in chloroform–methanol (19 : 1). The corresponding zone was extracted with ethyl acetate, solvent was removed and the product was crystallised from acetone to yield 15 mg of the diacetate *XI*, m.p. 194–195°C. Mass spectrum: M^+ 432.

3β,11β,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one (*XIII*)

The triol *XII* (500 mg) in dimethyl sulphoxide (25 ml) was submitted to hydroxylation with *Beauveria bassiana* as described for the diol *I*. The starting steroid disappeared after 24 to 30 h. The mycelium was filtered off, washed with three portions of ethyl acetate 500 ml each and the filtrate was extracted with the same solvent. The combined extracts were washed with 5% sodium hydrogen carbonate, water, dried and the solvent distilled off under reduced pressure. To remove the lipophilic components the residue was washed well with light petroleum. This crude reaction mixture (450 mg) consisted according to the TLC of three products of hydroxylation . They were separated on a silica gel column (100 g) in chloroform–methanol (19 : 1). Fractions containing the lipophilic component were worked up to yield 50 mg of a product contaminated with dimethyl sulphoxide. Repeated purification by preparative TLC in chloroform–methanol (9 : 1)

and crystallisation from ethyl acetate afforded 30 mg of the tetrol *XIII*, m.p. 220–221°C, $[\alpha]_D^{20}$ –2° (*c* 0·96). Mass spectrum: M^+ 350. ^{1}H-NMR: 0·76 (s, 18-H), 1·01 (s, 19-H), 4·18 (m, W = 8 Hz, 11α-H), 4·11 and 4·54 (2 d, J = 19 Hz, 21-H), 4·95 (s, 17-OH), 5·28 (s, 6-H), 3·32 (m, W = 30 Hz, 3α-H). For $C_{20}H_{30}O_5$ (350·4) calculated: 68·54% C, 8·63% H; found: 68% C, 8·61% H.

3β,11β,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one 3,21-diacetate (*XIV*)

The tetrol *XIII* (3 mg) in pyridine (0·4 ml) was acetylated with acetic anhydride (0·2 ml) at room temperature for 18 h. The anhydride was decomposed with saturated sodium chloride solution and the product isolated with ethyl acetate. The residue was crystallised from acetone to yield 2 mg of the diacetate *XIV*, m.p. 156–157°C, mass spectrum: M^+ 434.

17,20; 20,21-Bis(methylendioxy)-B-norpregn-5-en-3β,11β-diol (*XV*)

Paraformaldehyde (500 mg) in water (1·5 ml) and conc. hydrochloric acid (1·5 ml) was agitated at room temperature for 3 h. The tetrol *XIII* (40 mg) in dichloromethane (2 ml) was then added and agitated for 1 h. The mixture was treated with saturated sodium chloride solution (5 ml) and the product extracted with ethyl acetate. The extract was washed with 2% potassium carbonate with saturated sodium chloride solution, dried and the solvent removed. The residue (42 mg) was purified by preparative TLC in benzene–ether (1 : 1). The corresponding zone was extracted with chloroform to yield 19 mg of a product which on crystallisation from ethyl acetate–hexane gave 12 mg of the bis(methylendioxy) derivative *XV*, m.p. 230–231°C, $[\alpha]_D^{20}$ –81° (*c* 0·58 in chloroform). The compound was identical in all respects with the authentic compound prepared previously[4].

3β,15α,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one (*XVI*)

Elution of the chromatography after isolation of the tetrol *XIII* with chloroform–methanol (9 : 1) yielded fractions with the second product of hydroxylation. Working up gave 80 mg of a gumy residue containing some dimethyl sulphoxide. It was purified by preparative TLC in chloroform–methanol (9 : 1) and finaly chromatographed on a silica gel column (10 g) in chloroform–methanol (9 : 1) Working up of the corresponding fractions and crystallisation from ethyl acetate afforded 40 mg of the tetrol *XVI*, m.p. 238–239°C, $[\alpha]_D^{20}$ –12° (*c* 1·28). Mass spectrum: M^+ 350. ^{1}H-NMR: 0·66 (s, 18-H), 0·83 (s, 19-H), 3·01 (dd, $J_{16,16}$ = 15 Hz, $J_{16,15}$ = 9 Hz, 16-H), 4·13 and 4·55 (2d, J = 19 Hz, 21-H), 5·59 (s, 6-H), 3·29 (m, W = 30 Hz, 3α-H), 3·88 (*m*, 15β-H). For $C_{20}H_{30}O_5$ (350·4) calculated: 68·54% C, 8·63% H; found: 68·30% C, 8·52% H.

3β,15α,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one 3,15,21-triacetate (*XVII*)

The tetrol *XVI* (3 mg) in pyridine (0·4 ml) was acetylated with acetic anhydride (0·2 ml) for 18 hours at room temperature. Usual working up afforded 4 mg of the triacetate *XVII* which resisted all attempts at crystallisation. Mass spectrum: M^+ 476.

3β,15α-Dihydroxy-B-norandrost-5-en-17-one (*XVIII*)

A solution of the tetrol *XVI* (200 mg) in 50% acetic acid (6 ml) was treated with sodium bismuthate (2·5 g) and agitated for 3 h at room temperature. The mixture was diluted with ethyl acetate and water, the upper layer was washed with hydrochloric acid and worked up. The residue was purified by preparative TLC in chloroform–methanol (9 : 1). The corresponding zones were

collected, extracted with ethyl acetate and the solvent was removed. The residue was crystallised from acetone to yield 30 mg of the diol *XVIII*, m.p. 252–255°C, $[\alpha]_D^{20}$ −17° (*c* 0·93). Mass spectrum: M^+ 290. ^{1}H-NMR: 0·87 (s, 18 and 19-H), 1·90 and 2·81 (2 dd, 16-H), 4·23 (bm, 15-H), 4·54 (bs, OH), 4·88 (bd, OH), 5·65 (bs, 6-H); after addition of CD_3COOD: 0·87 (s, 18 and 19-H), 1·90 and 2·79 (2 dd, $J_{16,16}$ = 19 Hz, $J_{16,5}$ = 6·5 Hz + 8 Hz, 16-H), 3·34 (bs, 6-H), 4·21 (m, $J_{15,14}$ = 9 Hz, $J_{15,16}$ = 8 Hz + 6·5 Hz). For $C_{18}H_{26}O_3$ (290·4) calculated: 74·44% 9·03% H; found: 74·22% C, 9·10% H.

3β,11α,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one (*XIX*)

Elution of the chromatography after isolation of the tetrols *XIII* and *XVI* with the same solvent mixture afforded fractions with the most polar product of hydroxylation. Working up afforded 150 mg of a solid which was purified by preparative TLC (chloroform-methanol 9 : 1) and then by column chromatography on silica gel (15 g) in chloroform–methanol (9 : 1). The corresponding fractions were worked up and the residue was crystallised from ethyl acetate to yield 100 mg of the tetrol *XIX*, m.p. 216–218°C. $[\alpha]_D^{20}$ −58° (*c* 1·28). Mass spectrum: M^+ 350. ^{1}H-NMR: 0·57 (s, 18-H), 0·96 (s, 19-H), 4·17 and 4·58 (2 d, *J* = 19 Hz, 21-H), 5·04 (s, 17α-OH), 5·25 (s, 6-H), 3·33 (m, *W* = 30 Hz, 3α-H), 3·83 (m, *W* = 25 Hz, 11β-H). For $C_{20}H_{30}O_5$ (350·4) calculated: 68·54% C, 8·63% H; found: 68·22% C, 8·63% H.

3β,11α,17α,21-Tetrahydroxy-B-norpregn-5-en-20-one 3,11,21-triacetate (*XX*)

The tetrol *XIX* (3 mg) in pyridine (0·4 ml) was acetylated with acetic anhydride (0·2 ml) as described for the diacetate *III*. Similar working up and crystallisation from acetone gave 2 mg of the acetate *XX*, m.p. 191–192°C. Mass spectrum: M^+ 476.

3β,11α-Dihydroxy-B-norandrost-5-en-17-one (*XXI*)

A solution of the tetrol *XIX* (200 mg) in 50% acetic acid (6 ml) was treated with sodium bismutate (2·5 g) and agitated at room temperature for 3 h. The mixture was worked up as described for the dione *IV*. The ethyl acetate extract was reduced to 2 ml and allowed to crystallise to yield 30 mg of the diol *XXI*. The mother liquors were purified by preparative TLC in chloroform–methanol (9 : 1) to yield after working up and crystallisation additional 15 mg of the diol *XXI*, m.p. 116 to 124°C, $[\alpha]_D^{20}$ −80° (*c* 1·02), identical in all respects with the authentic[3] sample.

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