# 6β-Hydroxylation of 17α-Acetoxycortexone by *Flavobacterium*

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Fermentation of a mixture of 3,17α,21-triacetoxy-3,5-pregna-diene-20-one and 17α,21-diacetoxy-4-pregnene-3,20-dione with *Flavobacterium dehydrogenans* ATCC 13930 led to 17α-acetoxy-21-hydroxy-4-pregnene-3,20-dione as the main product and the novel 17α-acetoxy-6β,21-dihydroxy-4-pregnene-3,20-dione and 17α-acetoxy-6α,21-dihydroxy-4-pregnene-3,20-dione as the minor ones. 6-Hydroxylation was not found so far with this strain. The finding is in good accordance with the model of 6-hydroxylation of steroids proposed for fungi by Holland *et al.*

The production of important corticosteroids of high therapeutical activity from Reichstein S or its derivatives requires hydroxylation at 11β-position. This can be done by fermentation with *Curvularia lunata.* Unfortunately our starting material, a 1:1 mixture of 3,17α,21-triacetoxy-3,5-pregna-diene-20-one (**1**) and 17α,21-diacetoxy-4-pregnene-3,20-dione (**2**) is a rather bad substrate for this fungus so we used *Flavobacterium dehydrogenans* ATCC 13930 for a partial saponification of the mixture yielding Reichstein-S-17α-acetate (**3**). This product can then rapidly be hydroxylated by *Curvularia lunata* in high yields.

Main compound of the biotransformation products of **1** and **2** with *Flavobacterium dehydrogenans* ATCC 13930 is 17α-acetoxy-21-hydroxy-4-pregnene-3,20-dione (**3**) in 94% yield [1]. In minor amounts a more polar product was isolated which proved to be a 2:1 mixture of epimeric alcohols. Both compounds displayed doublets in the $^{13}C$ NMR spectra at $\delta_C$ 72.4 and 68.0 indicating the presence of secondary hydroxy groups. Compared to compound **3** the C-8 resonance is shielded from $\delta_C$ 35.6 to $\delta_C$ 29.8 and $\delta_C$ 29.6 respectively, the triplet at $\delta_C$ 31.8 (C-6) is missed and the resonance of C-7 is deshielded. These differences can only be explained by a hydroxy group at C-6. This finding was corroborated by the oxidation of the epimeric mixture with mangandioxide. The oxidation product **6** showed a bathochromic shift of its absorption maximum of 28 nm indicating the formation of the $\Delta^4$-3,6-dione. The coplanar α-hydroxy group at C-6 causes a much stronger shielding of C-4 than the β-hydroxy group while the β-hydroxy group has a pronounced δ-effect on C-19 due to its axial position. These assignments led to the conclusion that the main hydroxylation product is 17α-acetoxy-6β,21-dihydroxy-4-pregnene-3,20-dione (**4**) while 17α-acetoxy-6α,21-dihydroxy-4-pregnene-3,20-dione (**5**) is the minor one.

To our knowledge neither these metabolites nor the 6-hydroxylation of 4-pregnene-2-one derivatives with *Flavobacterium dehydrogenans* was reported so far [2]. 6β-Hydroxylation of 17α,21-dihydroxy-4-pregnene-3,20-dione and 17α-acetoxy-21-hydroxy-4-pregnene-3,20-dione with *Curvularia geniculata, C. lunata* and *C. inaequalis* was published by a Russian group [3]. De Rosa and colleagues

Table I. $^1H$ NMR data of biotransformation products **3–5** (400 MHz).

| | **3** $CDCl_3$ | **4** $CDCl_3$ | $C_5D_5N$ | **5** $CDCl_3$ | $C_5D_5N$ |
|---|---|---|---|---|---|
| 2-H | 2.83 ddd | 2.85 dd, br. | 3.10 | 2.85 dd, br. | 3.10 |
| 4-H | 5.74 d | 5.81 s | 6.02 | 6.20 s | 6.80 |
| 6-H | | 4.35 m | 4.51 | 4.35 m | 4.51 |
| 18-H | 0.67 s | 0.71 s | 0.75 | 0.68 s | 0.72 |
| 19-H | 1.20 s | 1.38 s | 1.48 | 1.18 s | 1.07 |
| 21-H | 4.27 m | 4.27 m | 4.66 | 4.27 m | 4.66 |
| OAc | 2.09 s | 2.09 s | | 2.10 s | |

*J*(Hz): **3:** 1,2 = 2,2′ = 16; 4,6 = 1.2; **4:** 1,2 = 2,2′ = 16; **5:** 1,2 = 2,2′ = 16; 4,6 = 1.5.

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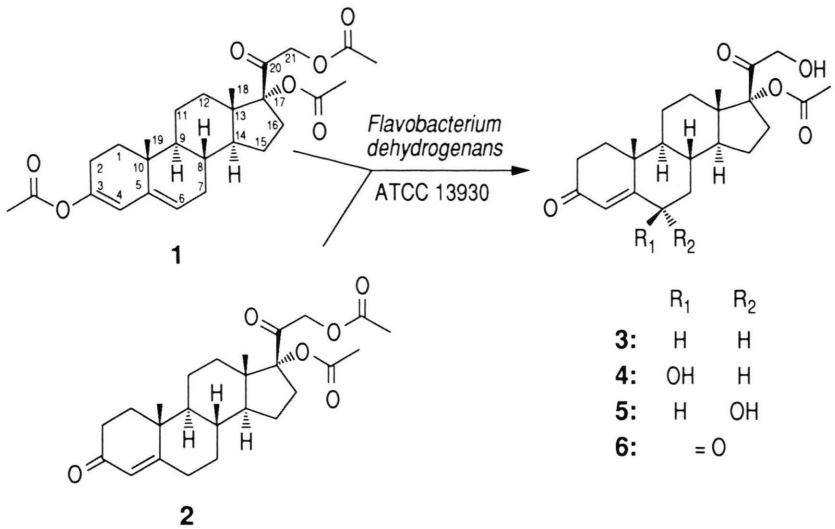


Fig. 1. 17α,21-Diacetoxy-pregnene-20-one derivatives **1** and **2** and its biotransformation products **3–5.**

Table II. $^{13}C$ NMR of Reichstein-S derivatives ($CDCl_3$, 25.1 MHz).

| | 3 | 4 | 5 |
|---|---|---|---|
| C-1 | 35.5 t | 36.9 t | 36.1 t |
| C-2 | 33.9 t | 34.0 t | 34.0 t |
| C-3 | 199.3 s | 200.5 s | 199.5 s |
| C-4 | 124.0 d | 126.1 d | 119.8 d |
| C-5 | 170.4 s | 168.3 s | 171.6 s |
| C-6 | 31.8 t | 72.4 d | 68.0 d |
| C-7 | 32.6 t | 37.8 t | 38.8 t |
| C-8 | 35.6 d | 29.8 d | 29.6 d |
| C-9 | 53.0 d | 52.8 d | 52.8 d |
| C-10 | 38.5 s | 38.3 s | 40.8 s |
| C-11 | 20.4 t | 20.3 t | 20.3 t |
| C-12 | 30.4 t[a] | 30.2 t[b] | 30.8 t[c] |
| C-13 | 47.6 s | 47.6 s | 47.5 s |
| C-14 | 50.8 d | 50.7 d | 50.4 d |
| C-15 | 23.8 t | 23.7 t | 23.7 t |
| C-16 | 30.6 t[a] | 30.5 t[b] | 30.5 t[c] |
| C-17 | 94.0 s | 94.0 s | 93.9 s |
| C-18 | 14.2 q | 14.1 q | 14.1 q |
| C-19 | 17.3 q | 19.3 q | 18.1 q |
| C-20 | 206.2 s | 206.1 s | 206.0 s |
| C-21 | 66.9 t | 66.8 t | 66.8 t |
| OAc | 170.8 s | 170.8 s | 170.8 s |
| | 21.1 q | 21.0 q | 21.0 q |

[a,b,c] Assignments may be interchanged.

reported on the 6-hydroxylation of 4-pregnene-3,20-dione with the extreme thermophilic bacterium *Caldariella acidophila* leading to 6α- and 6β-hydroxy-4-pregnene-3,20-dione [4]. Stereoselective 6β-hydroxylation of $C_{21}$-steroids was obtained with *Bacillus cereus* [5] and the fungi *Syncephalastrum racemosum* [6] and *Rhizopus arrhizus* [7].

In a series of publications Holland and coworkers reported on the mechanism of steroid-hydroxylations in *Rhizopus arrhizus* [8]. They found that in this fungus the active enzyme is a cytochrome P-450-dependent monooxygenase and that in 3-keto-$\Delta^4$-steroids the oxidation at C-6 proceeds *via* a 3,5-dienol intermediate [9]. Our results point to a similar or identical mechanism in bacteria which was found by Holland in fungi. A further confirmation for this mechanism comes from the fact that a 6-hydroxylation was not found in the biotransformation of 3α,17α,21-triacetoxy-pregn-5-ene-20-one with *F. dehydrogenans* [10].

## Experimental

### *Chemicals*

3,17α,21-triacetoxy-4-pregnene-20-one (Reichstein's substance S triacetate) and 17α,21-diacetoxy-4-pregnene-3,20-dione (Reichstein's substance S diacetate) in 1:1 mixture were obtained from Vlahov *et al.* [11], 17α,21-dihydroxy-4-pregnene-3,20-dione (Reichstein's substance S) from Sigma (Munich, F.R.G.). Powdered yeast extract was purchased from Ohly (Hamburg, F.R.G.), peptone-type N–Z case from Sheffield (England), antifoam AC 3368 from Bayer (Leverkusen, F.R.G.). All

other chemicals were obtained from Merck (Darmstadt, F.R.G.).

*Instruments used*

Fermentor: 10 l and 200 l fermenters were from Giovanola Freres S.A. (Monthey, Switzerland). The optical density of the cultures were measured at 546 nm in an Eppendorf photometer, out-gases were determined with a gas analyzer from Maihak (Hamburg, F.R.G.) and the glucose concentration was determined with a Beckman glucose analyzer. NMR: The $^1H$ NMR spectra were obtained at 400 MHz and the $^{13}C$ NMR spectra at 75.5 MHz, $CDCl_3$ was the solvent and TMS the internal standard. Mass spectra were recorded with 70 eV. IR spectra were measured in $CHCl_3$. Melting points are uncorrected.

*Cultivation procedures*

The bacterial strain *Flavobacterium dehydrogenans* ATCC 13930 was maintained on 1% of glucose, 0.5% of peptone, 1% of yeast extract and 2% agar at pH 6.5 and only 24 h old cultures were used for submerged cultivation. The medium for fermentation was 3% yeast extract, 0.17% $KH_2PO_4$, 0.22% $Na_2HPO_4$ at pH 6.8. The flasks (2 l) were inoculated with a physiological solution of the cell suspension. After 48 h cultivation at 30 °C at a rotary shaker with 250 rpm, the bioreactor was inoculated with 5% inoculation material. The fermentation parameters were as follows: 400 rpm (10 l fermenter) or 250 rpm (200 l fermenter), 30 °C, aeration 1 vvm. After 12 h the substrate (20 g/l) suspended in water was added under sterile conditions at the stationary phase and the pH was maintained at 6.0–6.5 by 5% solution of $NH_3$. Samples were taken each day and analyzed as follows: To 1 ml of culture broth 0.2 ml of EtOAc was added and shaken for 2 min prior to centrifugation. 10 µl of the extract was developed on HPTLC plates with $CH_2Cl_2$–$Me_2CO$ 9:1. The spots were made visible by spraying with anisaldehyde–$H_2SO_4$ in HOAc and heating to 110 °C for 1 min.

*Extraction and purification*

Culture medium and mycelia were separated by filtration and both were extracted three times with EtOAc. The solvent was evaporated and the crude extract crystallized or separated on Si-60 columns with a *n*-hexane/ethyl acetate gradient (changing from 9:1 to pure ethyl acetate). When necessary the collected fractions were further purified by prep. TLC.

Fermentation of **1** and **2** (1:1) (200 g) yielded after 24 h **3** (161 g), **4** (3.58 g) and **5** (1.79 g).

17α-Acetoxy-6β,21-dihydroxy-4-pregnene-3,20-dione (**4**) and 17α-acetoxy-6α,21-dihydroxy-4-pregnene-3,20-dione (**5**): Not completely separated mixture. M.p. 190 °C (decomp.), UV (MeOH): 239 nm. IR: 3600, 1740, 1720, 1680, 1620 $cm^{-1}$. MS *(m/z):* 404 (2%) ($M^+$), 373 (13), 344 (20), 329 (11), 326 (17), 285 (77), 267 (70), 43 (100). 1 mg of the mixture of **4** and **5** was solved in 2 ml diethylether, 2 mg of activated mangandioxide added and the slurry stirred for 30 min. After filtration an UV maximum at 267 nm (MeOH) was observed, indicating the formation of 17α-acetoxy-4-pregnene-3,6,20-trione (**6**).

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