

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

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Z. Naturforsch. **48c**, 727–730 (1993); received June 21, 1993

Microbial 6β-Hydroxylation, Steroid, *Flavobacterium dehydrogenans*, 17α-Acetoxycortexone, ¹³C NMR

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 ¹³C NMR spectra at δ_C 72.4 and 68.0 indicating the presence of secondary hydroxy groups. Compared to compound **3** the C-8 resonance is shielded from δ_C 35.6 to δ_C 29.8 and δ_C 29.6 respectively, the triplet at δ_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 mangan dioxide. The oxidation product **6** showed a bathochromic shift of its absorption maximum of 28 nm indicating the formation of the Δ⁴-3,6-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	4	5
CDCl ₃	CDCl ₃	C ₃ D ₃ N
2-H 2.83 ddd	2.85 dd, br.	3.10
4-H 5.74 d	5.81 s	6.02
6-H	4.35 m	4.51
18-H 0.67 s	0.71 s	0.75
19-H 1.20 s	1.38 s	1.48
21-H 4.27 m	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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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/0900–0727 \$ 01.30/0



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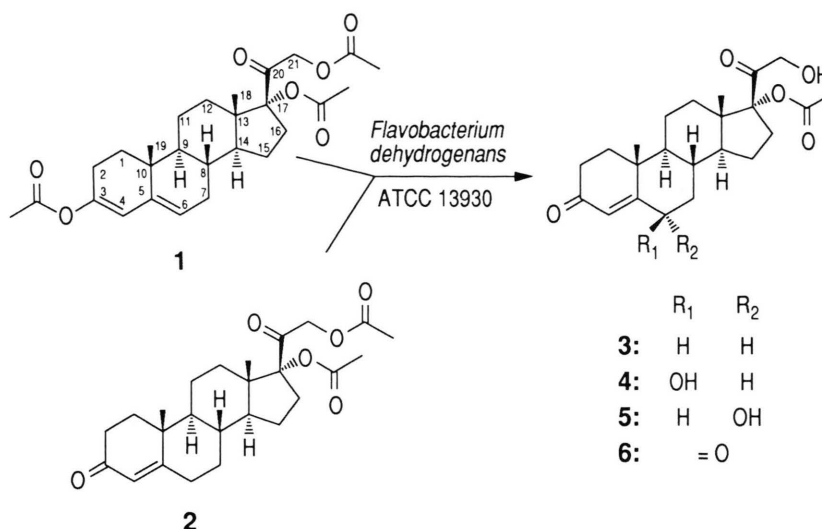


Fig. 1. 17 α ,21-Diacetoxy-pregnene-20-one derivatives **1** and **2** and its biotransformation products **3–5**.

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 6 α - and 6 β -hydroxy-4-pregnene-3,20-dione [4]. Stereoselective 6 β -hydroxylation of C₂₁-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- Δ^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 3 α ,17 α ,21-triacetoxy-pregn-5-ene-20-one with *F. dehydrogenans* [10].

Experimental

Chemicals

3,17 α ,21-triacetoxy-4-pregnene-20-one (Reichstein's substance S triacetate) and 17 α ,21-diacetoxy-4-pregnene-3,20-dione (Reichstein's substance S diacetate) in 1:1 mixture were obtained from Vlahov *et al.* [11], 17 α ,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).

17 α -Acetoxy-6 β ,21-dihydroxy-4-pregnene-3,20-dione (**4**) and 17 α -acetoxy-6 α ,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 17 α -acetoxy-4-pregnene-3,6,20-trione (**6**).

Acknowledgements

This work was supported in part by the Alexander von Humboldt-Stiftung, Bonn.

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