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Microbial hydroxylation of 13β-ethyl-4-gonene-3,17-dione

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Abstract

Among the microbiological transformations of steroids 15α-hydroxylation of 13β-ethyl-4-gonene-3,17-dione is an industrially important one [Von H. Hofmeister, K. Annen, H. Laurent, K. Petzoldt, R. Wiechert, Arzneim.-Forsch. 36 (1986) 781], since it results in an intermediate of the synthesis of Gestoden, a widely used contraceptive drug. The aim of our research was to select fungal strains for hydroxylation of 13β-ethyl-4-gonene-3,17-dione which produce the 15α-hydroxylated product in a high yield. According to our taxonomical studies, several species of *Aspergillus*, *Fusarium*, *Mortierella*, and *Penicillium* genera fulfill this requirement. It has been reported that the 15α-hydroxylating enzyme of *Penicillium raistrickii* is inducible by various steroidal compounds [S. Irrgang, D. Schlosser, H.-P. Schmauder, Biotechnol. Lett. 14 (1992) 33]. We found that the enzyme of *Fusarium nivale* (VJ-63 strain) is also advantageously induced by norethisterone, which significantly increased the economic efficiency of this biotransformation process [A. Jekkel, É. Ilkőy, J. Sütő, G. Ambrus, Gy. Horváth, I. Bősinger, I. Pallagi, I. Láng, E. Gyepessy, Hungarian Patent Appl. P-9602249 (1996)].

Keywords: Steroid hydroxylation; *Fusarium nivale*; 15α-Hydroxylation

1. Introduction

In the industrial synthesis of steroid drugs microbiologically hydroxylated steroid derivatives are often advantageously applied as intermediates. While every site in a steroid molecule is accessible for microbial hydroxylation, the 11α-, 11β-, 15α- and 16α-hydroxylations are accomplished now in the steroid industry mainly by microbial transformations. Two widely used contraceptive drugs: Gestoden (17α-ethynyl-13β-ethyl-17-hydroxy-4,15-gonadien-3-one) and Desogestrel (17α-ethynyl-13β-ethyl-11-methylene-4-gonen-17-ol) are manufactured using 15α-hydroxy- and 11α-hydroxy-13β-ethyl-4-gonene-3,17-dione, as the key intermediates, respectively. The aim of our research was to select fungal strains which convert 13β-ethyl-4-gonene-3,17-dione into its 15α-hydroxy derivative in a high yield and to study the byproducts accumulated during the 15α-hydroxylation process. In our experiments 3000 fungal strains, selected from the strain collection of the Institute for Drug Research, Budapest or isolated from natural habitats were investigated for their ability to hydroxylate 13β-ethyl-4-gonene-3,17-dione.

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2. Experimental

2.1. Screening of 15α-hydroxylating fungi

The fungal strains were cultivated on agar slants containing malt extract–yeast extract (Difco). The 13β-ethyl-4-gonene-3,17-dione bioconversions were carried out in 500 ml flat-bottomed flasks containing 50 ml culture medium consisting of 1% glucose, 3% malt extract (Difco), 0.3% yeast extract (Difco). After cultivation at 25°C for 24 hours on a rotary shaker (deflection 2.5 cm, 320 rotation min^{-1}) 200 mg 13β-ethyl-4-gonene-3,17-dione in 1 ml acetone were added to the shaken cultures, and cultivation was continued for 4 days. The microbial conversion of 13β-ethyl-4-gonene-3,17-dione was monitored by TLC and HPLC. For the TLC analysis we used silica gel chromatoplates (Merck, Alufoil DC_{254}) and ethanol–chloroform (5:95) and ethyl acetate–cyclohexane–ethanol–chloroform (60:50:5:0.3) mixtures, as developing solvents. For the HPLC analysis the broth samples were diluted tenfold with methanol, then centrifuged and the supernatants were used for assay (HPLC apparatus: LKB system: guard column Nucleosil C_8 10 μm (BST, Budapest, Hungary)—40 mm × 4 mm and analytical column Nucleosil C_{18} 10 μm (BST, Budapest, Hungary)—250 mm × 4 mm; temperature: 20°C; detection at 238 nm; eluent A: 5% acetonitrile, 95% water; eluent B: 100% acetonitrile; linear gradient, flow rate: 1 ml/min; injection volume: 10 μl).

2.2. Isolation and structural investigation of the biotransformation products

After fermentation the culture was filtered, and the transformation products were extracted exhaustively with ethyl acetate. The transformation products in the evaporation residues of ethyl acetate extracts were separated by silica gel column chromatography. The silica gel column was eluted with ethyl acetate–hexane mixtures with gradually increasing ethyl acetate content. The structures of the transformation products were elucidated by UV-, IR-, NMR- and mass spectroscopy [1].

Characteristic spectral data of compounds II to V: NMR spectra were measured in $CDCl_3$ on a Bruker AC 250 instrument using tetramethylsilane (TMS) as the reference standard. Mass spectra were taken on a Finnigan MAT 8430 instrument under the following operating conditions: resolution, 1250; ion accelerating voltage, 3 kV; electron energy, 70 eV; electron current, 500 μA; ion source temperature, 250°C; individual sample evaporation temperatures, between 140 and 180°C. Elemental compositions of ions were determined by high resolution mass measurements using perfluorokerosene (PFK) as the reference standard.

Compound II (m.p. 178–180°C): ^{1}H-NMR data (δ [ppm]): H-4, 5.85; H-15β, 4.50. ^{13}C-NMR data (δ [ppm]): C-4, 124.6; C-5, 165.9; C-15, 69.4; C-17, 214.9. MS data (EI): $M^{+\cdot}$ 302 (100%, $C_{19}H_{26}O_3$); *m/z* 274 (54%, $C_{17}H_{22}O_3$); *m/z* 260 (13%); *m/z* 231 (17%, $C_{16}H_{23}O$ and $C_{15}H_{19}O_2$); *m/z* 201 (14%, $C_{14}H_{17}O$); *m/z* 110 (58%).

Compound III (m.p. 235–240°C): ^{1}H-NMR data (δ [ppm]): H-7 α, 3.60; H-15 β, 4.58. ^{13}C-NMR data (δ [ppm]): C-4, 125.6; C-7, 71.8; C-15, 68.7; C-17, 214.8. MS data (EI): $M^{+\cdot}$ 318 (52.5%, $C_{19}H_{26}O_4$); *m/z* 300 (5%,); *m/z* 290 (10%, $C_{18}H_{26}O_3$); *m/z* 262 (14%, $C_{16}H_{22}O_3$); *m/z* 110 (100%, $C_7H_{10}O$).

Compound IV (m.p. 202–206°C): ^{1}H-NMR data (δ [ppm]): H-4, 5.78. ^{13}C-NMR data (δ [ppm]): C-4, 124.3; C-5, 164.0; C-10, 70.2. MS data (EI): $M^{+\cdot}$ 302 (84%, $C_{19}H_{26}O_3$); *m/z* 274 (100%, $C_{18}H_{26}O_2$); *m/z* 245 (42%, $C_{16}H_{21}O_2$); *m/z* 108 (85%); *m/z* 99 (82%, $C_5H_7O_2$).

Compound V (m.p. 214–216°C): ^{1}H-NMR data (δ [ppm]): H-4, 5.93; H-6α, 4.45. ^{13}C-NMR data (δ [ppm]): C-4, 125.6; C-5, 164.7; C-6, 71.5. MS data (EI): $M^{+\cdot}$ 302 (100%, $C_{19}H_{26}O_3$); *m/z* 284 (4%,); *m/z* 273 (13%); *m/z* 258 (10%, $C_{17}H_{22}O_2$); *m/z* 138 (28%, $C_8H_{10}O_2$).

3. Results and discussion

In the bioconversion of 13β-ethyl-4-gonene-3,17-dione (I) carried out with species of *Fusarium* genus, *Fusarium nivale* has been selected for introduction of a hydroxyl group into the 15α-position of 13β-ethyl-4-gonene-3,17-dione. We found that the 15α-hydroxylase of *F. nivale* strains was inducible by norethisterone applied in 0.25 g/l concentration during the growth of biomass in the seed culture. The conversion of compound I into its 15α-hydroxy-derivative II was increased effectively by strain selection, changes in media composition and variations in the parameters of bioconversion in laboratory fermentors.

Using *F. nivale* (VJ-63 strain) the yield of bioconversion for compound II was as high as 76.5% by adding compound I to the broth in a 4 g/l concentration. Besides compound II as the major product, the formation of the novel 7β,15α-dihydroxy-13β-ethyl-4-gonene-3,17-dione (III) was also observed from compound I. By studying the bioconversion of compound I with another *F. nivale* strain (VJ 90) compound II was also isolated as the major product and the hydroxylation at C-7 similarly occurred. Among the various *Fusarium* species studied *Fusarium moniliforme* proved to be a useful strain for 15α-hydroxylation of compound I too. In addition to *Fusaria*, several strains of species from *Aspergillus*, *Mortierella* and *Penicillium* genera accumulated the 15α-hydroxy derivative II from compound I. We studied in detail the bioconversion of compound I with *Mortierella pusilla*, where besides the 15α-hydroxylation the introduction of 6β- and 10β-hydroxyl groups also occurred.

The structures of biotransformation products of 13β-ethyl-4-gonene-3,17-dione produced by *F. nivale* and *M. pusilla* are shown in Fig. 1. Compounds II and IV were described previously, their structures have been confirmed by 1D and 2D NMR investigations as well as mass spectral fragmentation patterns. The positions and the configurations of the hydroxyl groups in

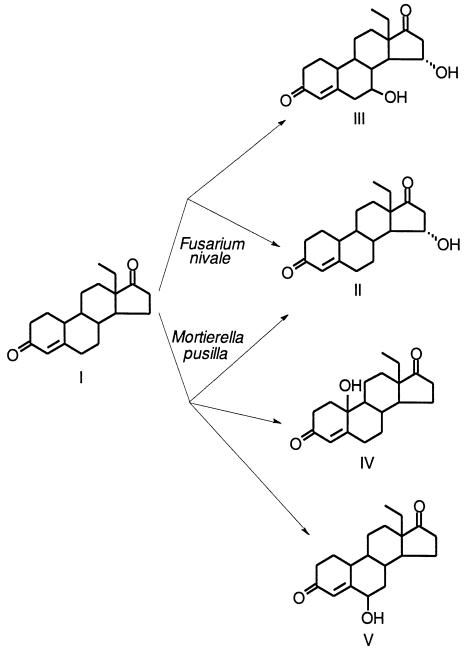

Fig. 1. Structures of the biotransformation products.

compound III were determined by using NOE experiment on both the 7α-H and the olefinic H, the irradiation of which resulted in an enhancement of the 6α proton, indicating the 7β-position of one of the hydroxyl groups. The 15α-position of the other hydroxyl group in compound III—similarly to compound II—has been determined in a ^{13}C-INEPT experiment showing correlation of the 15β-H with C-17 as well as from the coupling constant of that proton with 14α-H (10.1 Hz). The position and the configuration of the hydroxyl group in compound V were determined by using NOE experiment, where the irradiation of the 6α-H resulted in a 19% enhancement of the signal of the olefinic proton and from the small value of the coupling constants indicating its equatorial position. The mass spectral fragmentation patterns of compounds III and V are in accordance with the structures given.

References

[1] Gy. Horváth, I. Pallagi, É. Ilkőy, A. Jekkel, G. Ambrus, 6th Symposium on the Analysis of Steroids, Paper No. 53, Szeged, 7–9 October 1996.