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Steroids 70 (2005) 193–198
Steroids
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Steroids' transformations in *Penicillium notatum* culture

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Received 27 July 2004; received in revised form 19 November 2004; accepted 29 November 2004

Abstract

The application of *Penicillium notatum* genus for biotransformations of steroids has been investigated. The reactions observed include insertion of an oxygen atom into D-ring of steroids, 15α-hydroxylation of 17α-methyl testosterone derivatives, ester bond hydrolysis, and degradation of a testosterone derivatives side chain.

Microbial production of testolactones, the biologically active compounds, was also achieved using this strain in up to 98% yield.

Keywords: Biotransformation; Testolactone; *Penicillium notatum*; Steroids

1. Introduction

Microorganisms have found wide application in biotransformations of steroids in order to obtain more functionalized compounds due to high regio- and stereoselectivity of these reactions. Although many of such bioconversions are well known, there are ongoing efforts to increase their efficiency and to find new useful microorganisms and reactions. In recent years numerous research involving biotransformations of a wide range of steroid substrates have been reported [1–3]. Some of these biotransformations were carried out using strains belonging to the *Penicillia* genus, however, the attention was focused mainly on 15α-hydroxylation (catalyzed by *P. raistrickii* [4,5]) and 5α reductase enzyme of *P. decumbens* [6,7] and *P. crustosum* [8].

Having observed that testosterone **1** was metabolized by *Penicillium notatum* into a single product (testolactone **11**) in 98% yield, we decided to investigate the course of the transformations of other steroid substrates by means of this strain. The substrates that we selected for our study include: testosterone **1**, testosterone propionic ester **2**, androstenedione **3**, 19-nortestosterone **4**, progesterone **10**, 17α-ethynyltestosterone **8**, noretandrolon **9** and the three pharmacologically important testosterone derivatives with the 17α-methyl group and/or an additional double bond in the A-ring (17α-methyltestosterone **5**, dianabol **7**, 1-dehydrotestosterone **6**) (Fig. 1).

As far as biotransformations by *P. notatum* are concerned, we have not found in literature any reports on the correlation between a steroid substrate structure and the transformation profile. For this reason we have chosen for our research 10 substrates with various substituents at C-17, C-10 and in the A-ring.

2. Experimental

2.1. Microorganism

Classification of *P. notatum* KCH 904 was performed by Department of Phytopathology of Agriculture University of Wrocław. The organism was isolated from air.

2.2. Conditions of cultivation and transformations

The fungus was maintained on agar slants composed of aminobac (0.5%), peptone (0.5%), glucose (4%), agar (1.5%) (pH 5.9). Mycelium was used to inoculate 2-l flat-bottomed flasks, each containing 300 ml of the medium consisting of

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0039-128X/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2004.11.011

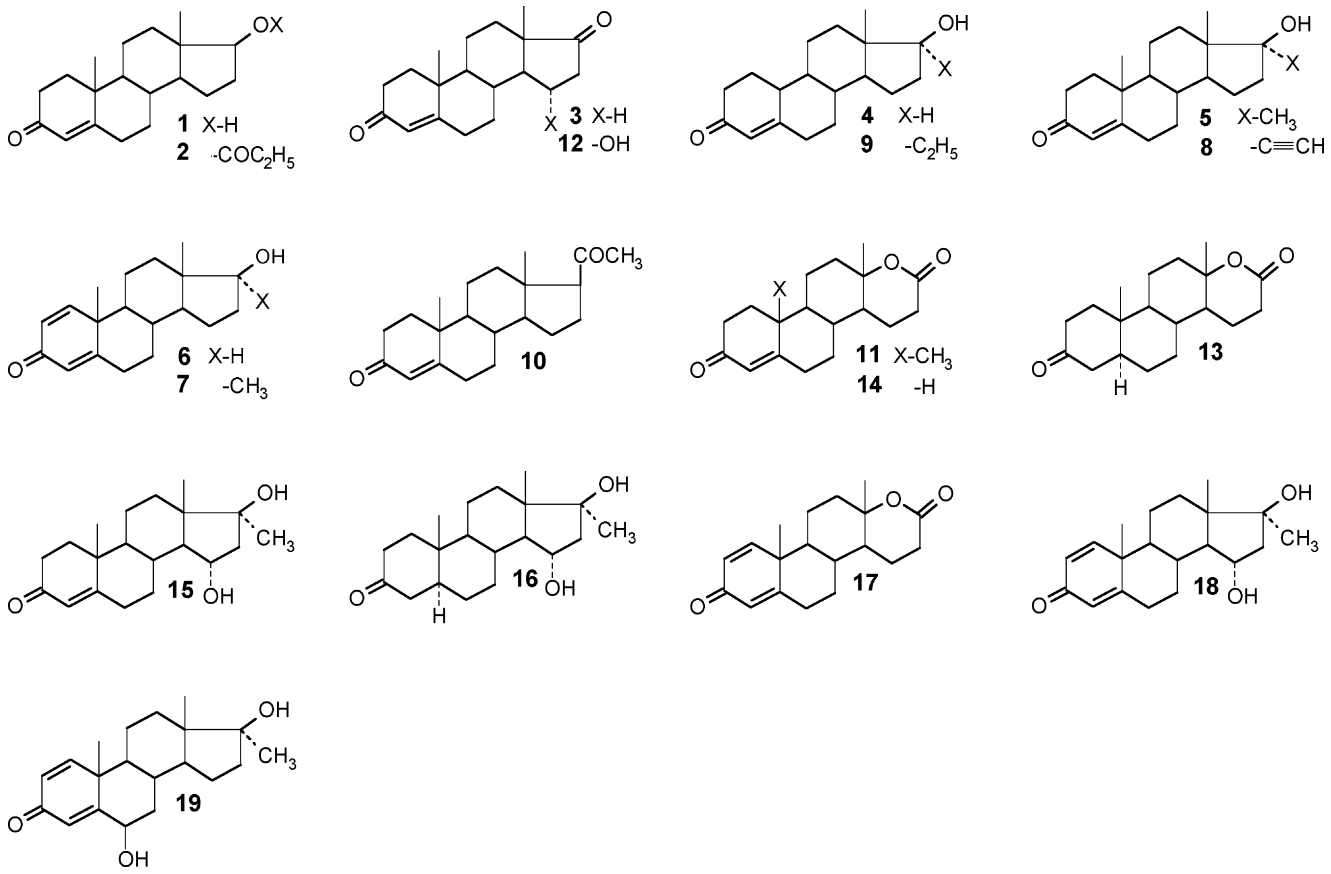

Fig. 1. Structures of substrates and products of biotransformations by *Penicillium notatum*.

3% glucose and 1% peptone. After cultivation at 27 °C for 48 h on a rotary shaker, 100 mg of a substrate, dissolved in 3 ml of acetone, was added. Biotransformation was carried out under the above conditions for further 2–14 days (until the substrate was metabolized, which was monitored chromatographically).

2.3. Product analysis

The transformation products were extracted from the fermentation media with chloroform and compositions of the crude mixtures were analyzed by TLC and GC.

The products were separated by column chromatography on silica gel (0.05–0.2 mesh, Merck) with hexane/acetone (2:1, v/v) as eluent. TLC analysis was carried out using commercially available plastic-backed plates pre-coated with a 0.2 mm layer of silica containing a fluorescent indicator (F_{254}, Merck), with hexane:acetone (2:1, v/v) as a developing solvent. Visualization of steroids was achieved by spraying the plates with ethanol:H_2SO_4 (1:1, v/v) and heating or by using UV lamp. GC analysis was performed using a Hewlett Packard 5890A (Series II) GC instrument fitted with a flame ionization detector (FID) and HP-5 column (cross-linked 5% Ph-Me-Silicone, 30 m × 0.53 mm × 0.88 μm film thickness), with N_2 as a carrier gas (2 ml/min flow rate).

The structures of biotransformation products were determined using spectral measurements. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a DRX 300 Bruker instrument in deuteriochloroform ($CDCl_3$) as a solvent and tetramethylsilane (TMS) as an internal standard. ^{1}H NMR data are given in Table 1.

Infrared (IR) spectra were recorded in KBr on a Mattson IR 300 spectrometer, and CD spectra in chloroform on a Jasco J-600 spectropolarimeter.

3. Results

3.1. Biotransformations of steroids in Penicillium notatum culture (^{1}H NMR data are given in Table 1)

3.1.1. 17β-Hydroxy-4-androsten-3-one (testosterone) **1**

The substrate **1** was converted into 17a-oxa-D-homo-4-androsten-3,17-dione (testolactone) **11** (98%) during 3 days.

^{13}C NMR: δ (ppm): 199.0 (C-3), 171.1 (C-17), 169.3 (C-5), 124.1 (C-4), 82.7 (C-13), 52.5 (C-9), 45.7 (C-14), 39.0 (C-12), 38.4 (C-10), 38.0 (C-8), 35.5 (C-1), 33.8 (C-2), 32.3 (C-7), 30.4 (C-6), 28.5 (C-16), 21.8 (C-11), 20.0 (C-18), 19.8 (C-15), 17.4 (C-19).

IR: ν_{max} (cm^{-1}): 1717, 1667, 1615, 1211, 1096.

Table 1
^{1}H NMR data for substrates and *Penicillium notatum* transformation products (chemical shift in ppm relative to TMS; solvent $CDCl_3$)

Compound	H-4	17α-H	17α-CH_3	H-18	H-19	C*H*—OH	Other significant signals
1	5.70	3.63, t, J = 8.5 Hz	–	0.77	1.17	–	–
2	5.72	4.60, t, J = 8.4 Hz	–	0.82	1.18	–	1.12, t, J = 7.6 Hz, 17β-O—CO—CH_2—CH_3
3	5.73	–	–	0.90	1.20	–	–
4	5.80	3.63, t, J = 8.7 Hz	–	0.78	–	–	–
5	5.72	–	1.20	0.90	1.19	–	–
6	6.04	3.61, t, J = 8.4 Hz	–	0.79	1.21	–	7.03, d, J = 10.1 Hz, H-1; 6.20, dd, J = 1.8, 10.1 Hz, H-2
7	6.05	–	1.18	0.92	1.24	–	7.04, d, J = 10.1 Hz, H-1; 6.21, dd, J = 1.9, 10.1 Hz, H-2
8	5.72	–	–	0.88	1.19	–	2.56, s, 17α-C≡C*H*
9	5.81	–	–	0.93	–	–	0.97, t, J = 7.3 Hz, 17α-CH_2CH_3
10	5.72	2.52, t, J = 9.0 Hz	–	0.65	1.17	–	2.11, s, H-21
11	5.72	–	–	1.33	1.14	–	–
12	5.73	–	–	0.94	1.21	4.41, dt, J = 3.4, 9.2 Hz, H-15β	3.00, dd, J = 8.0, 19.4 Hz, H-16β
13	–	–	–	1.31	0.97	–	–
14	5.83	–	–	1.35	–	–	–
15	5.72	–	1.34	0.90	1.20	4.09, dt, J = 3.4, 8.6 Hz, H-15β	–
16	–	–	1.33	0.86	1.02	4.05, dt, J = 3.4, 9.2 Hz, H-15β	–
17	6.05	–	–	1.35	1.18	–	7.01, d, J = 10.2 Hz, H-1; 6.22, dd, J = 1.9, 10.1 Hz, H-2
18	6.06	–	1.32	0.93	1.25	4.09, dt, J = 3.5, 9.2 Hz, H-15β	7.04, d, J = 10.1 Hz, H-1; 6.22, dd, J = 1.7, H-2
19	6.15	–	1.19	0.96	1.45	4.52, t, J = 2.9 Hz, H-6α	7.04, d, J = 10.1 Hz, H-1; 6.20, dd, J = 1.8, 10.1 Hz, H-2

*3.1.2. 17β-Hydroxy-4-androsten-3-one propionate (testosterone propionate) **2***

After 4 days of incubation the main product of biotransformation of **2** was testolactone **11** (61%). The minor metabolites identified include 15α-hydroxy-4-androsten-3,17-dione (15α-hydroxyandrostenedione) **12** (29%) and 17a-oxa-D-homo-5α-androstan-3,17-dione **13** (9%).

^{13}C NMR: δ (ppm): 211.2 (C-3), 171.4 (C-17), 83.1 (C-13), 52.5 (C-9), 46.1 (C-5), 45.9 (C-14), 44.3 (C-4), 39.2 (C-12), 38.2 (C-1), 37.9 (C-2), 37.8 (C-8), 35.6 (C-10), 30.3 (C-7), 28.6 (C-6), 28.5 (C-16), 22.2 (C-11), 20.1 (C-18), 19.8 (C-15), 11.3 (C-19).

CD: $[\Theta]_{293\,nm}$ (°): 4594.

*3.1.3. 4-Androsten-3,17-dione (androstenedione) **3***

After 3 days of incubation with substrate **3**, the same products as above were isolated and separated by column chromatography in the following yield: testolactone **11** (82%), 15α-hydroxyandrostenedione **12** (7%) and 17a-oxa-D-homo-5α-androstan-3,17-dione **13** (7%).

*3.1.4. 17β-Hydroxy-4-estren-3-one (19-nortestosterone) **4***

Transformation of **4** gave 17a-oxa-D-homo-4-estren-3,17-dione (19-nortestolactone) **14** (93%).

^{13}C NMR: δ (ppm): 199.2 (C-17), 171.0 (C-3), 164.6 (C-5), 124.6 (C-4), 82.7 (C-13), 48.1 (C-9), 44.8 (C-14), 42.4 (C-12), 42.1 (C-10), 38.7 (C-8), 36.2 (C-2), 34.9 (C-6), 29.3 (C-7), 28.3 (C-16), 26.9 (C-11), 26.1 (C-1), 19.9 (C-15), 19.6 (C-18).

IR: ν_{max} (cm^{-1}): 1729, 1656, 1611, 1209, 1108.

*3.1.5. 17β-Hydroxy-17α-methyl-4-androsten-3-one (17α-methyltestosterone) **5***

After 13 days of incubation the following products were obtained: 15α,17β-dihydroxy-17α-methyl-4-androsten-3-one (15α-hydroxy-17α-methyltestosterone) **15** (65%) and 15α,17β-dihydroxy-17α-methyl-5α-androstan-3-on **16** (31%).

^{13}C NMR: δ (ppm): 211.9 (C-3), 79.1 (C-17), 72.3 (C-15), 58.3 (C-14), 50.4 (C-16), 46.8 (C-13), 46.5 (C-5), 44.6 (C-4), 38.6 (C-1), 38.1 (C-2), 35.8 (C-8), 32.1 (C-6), 31.6 (C-7), 28.7 (C-12), 26.1 (17α-CH_3), 20.9 (C-11), 15.5 (C-19), 11.5 (C-18).

IR: ν_{max} (cm^{-1}): 3418, 1699.

CD: $[\Theta]_{293\,nm}$ (°): 4211.

*3.1.6. 17β-Hydroxy-1,4-androstadien-3-one (1-dehydrotestosterone) **6***

Transformation of **6** was carried out for four days and 17a-oxa-D-homo-1,4-androstadien-3,17-dione (1-dehydrotestolactone) **17** was the only product isolated from the chloroform extracts (90%).

^{13}C NMR: δ (ppm): 185.9 (C-3), 170.8 (C-17), 167.3 (C-5), 154.4 (C-1), 128.0 (C-2), 124.1 (C-4), 82.4 (C-13), 50.8 (C-9), 45.6 (C-14), 42.9 (C-10), 38.9 (C-12), 37.9 (C-8), 32.2 (C-7), 32.0 (C-6), 28.4 (C-16), 23.4 (C-11), 20.1 (C-15), 20.1 (C-18), 18.7 (C-19).

*3.1.7. 17β-Hydroxy-17α-methyl-1,4-androstadien-3-one (dianabol) **7***

After 11 days of incubation 15α,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (15α-hydroxydianabol) **18** (83%) was isolated as the main product of the biotransfor-

mation, along with the minor metabolite 6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (6β-hydroxydianabol) **19** (9%).

*3.1.8. 17α-Ethynyl-17β-hydroxy-4-androsten-3-one (17α-ethynyltestosterone) **8** and 17α-ethyl-17β-hydroxy-4-estren-3-one (noretandrolon) **9***

In both cases, after 14 days of incubation the crude reaction mixtures contained only unreacted starting materials.

*3.1.9. 4-Pregnen-3,20-dione (progesterone) **10***

Incubation substrate **10** with *P. notatum* was carried out for 2 days and gave testolactone **11** (94%) (see Section 3.1.4) as a single product.

3.2. Identification of products

Testolactone **11** was formed as a product of the biotransformations of the following four substrates: testosterone **1**, testosterone propionic ester **2**, androstenedione **3**, and progesterone **10**. The structure of this product was elucidated on the basis of spectral data.

Cleavage of the acyl side chain of progesterone **10** is proved by the absence of the characteristic H-21 singlet at $\delta = 2.11$ ppm in ^{1}H NMR spectrum of compound **11**. Unchanged signals of H-4 and H-19 ($\delta = 5.72$ and 1.14 ppm, respectively) and the absence of H-17 triplet, which are typical for androstenedione (**3**), indicate the presence of ketone group at C-17. Metabolite **11** is considerably more polar than substrates **1**, **2**, **3** and **10**, which together with the absence of midfield C*H*OH signals in its ^{1}H NMR spectrum suggests hydroxylation of the tertiary carbon. However, the singlet of H-18 shows a very distinctive shift to lowfield (by 0.43 ppm in relation to androstenedione **3**). It may be explained by the close proximity of the oxygen atom.

The infrared analysis of **11** showed two carbonyl absorption bands at $\nu = 1717\ cm^{-1}$ (C-17) and $1667\ cm^{-1}$ (C-3), along with the asymmetrical and symmetrical stretching bands of C–O–C bonds ($\nu = 1211$ and $1096\ cm^{-1}$, respectively). We did not observe a hydroxyl group signal.

In the ^{13}C NMR spectrum of **11** signals of 19 carbons are visible, while DEPT showed the presence of two methyl ($\delta = 20.0$ and 17.4 ppm), eight methylene ($\delta = 39.0$, 35.5, 33.8, 32.3, 30.4, 28.5, 21.8 and 19.8 ppm), four methine ($\delta = 124.1$, 52.5, 45.7 and 38.0 ppm), and five ($\delta = 199.0$, 171.1, 169.3, 82.7 and 38.4 ppm) quaternary carbon atoms. The signal assigned to the C-13 showed a significant downfield shift ($\delta = 82.7$ ppm) compared to the starting material.

All these data allowed us to identify the metabolite as testolactone **11**. The final confirmation of the structure was a comparison of its ^{1}H NMR spectral data with those reported in the literature [9].

*3.2.1. 15α-Hydroxyandrostenedione **12***

The ^{1}H NMR spectrum of **12** was similar to that of **3**, with the exception of a new signal at $\delta = 4.41$ ppm, which was ascribed to a methin proton bearing a hydroxyl group. The regio- and stereochemistry of the hydroxylation were established by the shape and multiplicity of the C*H*OH signal: a triplet split into doublet, which is characteristic for 15β protons [9]. The observed effect of the additional hydroxyl group on chemical shifts of protons H-18 ($\Delta\delta = 0.04$ ppm), H-19 ($\Delta\delta = 0.01$), and H-4 ($\Delta\delta = 0.0$ ppm) in comparison to **3**, and the presence of a signal of 16β proton at $\delta = 3.00$ ppm (dd, $J_{16\alpha16\beta} = 19.4$, $J_{15\beta16\beta} = 8.0$ Hz) confirmed this structure.

*3.2.2. 17a-oxa-D-homo-5α-androstan-3,17-dione **13***

The ^{1}H NMR spectrum of **13** bears resemblance to that of **11**, with the exception of H-19 singlet, which is shifted upfield ($\Delta\delta = 0.17$ ppm), and the disappearance of the olefinic proton signal due to the 5α reduction of the Δ^4 double bond.

Circular dichroism spectroscopy was used to confirm the 5α position of introduced H-5 and the spectrum of **13** in chloroform shows a positive Cotton effect at 293 nm (3-C=O).

The ^{13}C NMR spectrum of **13** reveals upfield shift of signals assigned to C-4 and C-5 ($\Delta\delta = 79.8$ and 123.3 ppm, respectively), compared to the **11** one.

Additionally, the spectral data of this compound closely correspond to the literature data for 17a-oxa-D-homo-5α-androstan-3,17-dione [10].

*3.2.3. 19-Nortestolactone **14***

In the ^{1}H NMR spectrum of **14** the signal of H-18 is strongly shifted towards lower field ($\Delta\delta = 0.57$ ppm) in comparison to the starting material. The H-17α triplet ($\delta = 3.63$ ppm), which is characteristic for 19-nortestosterone **4**, have disappeared, while the ^{13}C NMR spectrum of **14** shows a new carbonyl signal ($\delta = 199.2$ ppm). The signal of C-13 shows a significant downfield shift to $\delta = 82.7$ ppm, caused by the insertion of an oxygen atom into the D-ring.

The IR spectrum of compound **14** confirms the proposed structure. The features of note include absorption bands at $\nu = 1729\ cm^{-1}$ (17-C–O) and $\nu = 1611$ and $1656\ cm^{-1}$ for the Δ^4 and 3-C=O functionalities, respectively. The stretching bands of C–O–C are observed at $\nu = 1209$ and $1108\ cm^{-1}$.

*3.2.4. 15α-Hydroxy-17α-methyltestosterone **15** and 15α-hydroxydianabol **18***

Both position and configuration of a newly introduced 15α-hydroxyl group were recognized mainly by the characteristic shape (dt) of the signal at $\delta = 4.09$ ppm, and a significant downfield shift of 17α-CH_3 signal ($\Delta\delta = 0.14$ ppm) in the ^{1}H NMR spectrum.

*3.2.5. 15α,17β-Dihydroxy-17α-methyl-5α-androstan-3-on **16***

^{1}H NMR spectrum of compound **16** is similar to that of compound **15** except for the lack of H-4 singlet, and an upfield

shift of the H-19 signal ($\Delta\delta = 0.18$ ppm), which is characteristic for the 5α reduction of the double bond.

The CD spectrum shows a positive Cotton effect and the value of [Θ] at 293 nm (4211) confirmed this structure.

The IR spectrum indicates the presence of hydroxyl ($\nu = 3418\,cm^{-1}$) and ketone ($\nu = 1699\,cm^{-1}$) groups. ^{13}C NMR spectrum of **16** exhibits signals of 20 carbons. The additional hydroxyl-bearing methine carbon is characterized by the lowfield chemical shift ($\delta = 72.3$ ppm), whilst C-5 and C-4 carbons appears at $\delta = 46.5$ and 44.6 ppm, respectively.

*3.2.6. 1-Dehydrotestolactone **17***

Compared to the starting material **6**, the changes in chemical shifts of H-4 ($\Delta\delta = 0.01$ ppm), H-18 ($\Delta\delta = 0.56$ ppm), and H-19 ($\Delta\delta = 0.03$ ppm) signals in the ^1H NMR spectrum of **17** are the same as in the case of testosterone **1** conversion into testolactone **11**. In the ^{13}C NMR spectrum the new quaternary carbon signal of C-13 resonated at $\delta = 82.4$ ppm, which supported the structural assignment.

*3.2.7. 6β-Hydroxydianabol **19***

The 6β hydroxylation was distinguished by a large downfield shift ($\Delta\delta = 0.21$ ppm) of the H-19 singlet, and by the narrow triplet ($J_{gem} = 2.9$ Hz) profile of a signal at $\delta = 4.52$ ppm, ascribed to the equatorial 6α-proton. The change of H-4 chemical shift ($\Delta\delta = 0.10$ ppm) is also significant, as it places this signal in a position observed for 6β-alcohols, which confirms the presence of 6β-hydroxyl group.

4. Discussion

The aim of the present study was to investigate the utility of *P. notatum* for useful steroid transformations and to determine the influence of a substrate structure on the reaction route. In order to examine the effect of the additional $C_1{=}C_2$ double bond, the lack of 19-methyl group and the type of substituent at C-17, the courses of the transformations of 10 substrates were compared.

With the exception of 17α-ethynyltestosterone **8** and noretandrolon **9**, the rest of the substrates underwent complete transformations, with all the products obtained being more polar than the starting materials.

Conversion of the male hormone—testosterone **1** by *P. notatum* led to a single product—oxidized metabolite testolactone **11** in a very high yield (98%). Other substrates that were transformed in a similar way comprise: testosterone propionate **2**, androstenedione **3**, progesterone **10**, 1-dehydrotestosterone **6**, and 19-nortestosterone **4**. To sum up, it has been observed that the fungus can perform an oxygen atom insertion into the D-ring, an ester bond hydrolysis, a carbon-carbon bond scission in the side chain of progesterone derivative **10**, 17β-alcohol oxidation, 15α-hydroxylation and the Δ^4 reduction. Moreover, no influence of the additional double bond in the A-ring and of the lack of 19-methyl group on the course of the transformations has been noticed.

Testolactones are biologically important compounds, expressing biological activity of different kinds. Particularly interesting is their role of antiestrogens and anticarcinogens [11]. They are moderately specific first generation inhibitors of human aromatase activity and thereby may contribute to the prevention of hormone dependent tumors such as breast cancer [12], prostatic hyperplasia, and prostate cancer [13]. Testolactones are used as therapeutic agents in disorders caused by imbalance between estrogen and androgen action, e.g. gynecomastia [14] or precocious puberty [15]. Aromatase inhibitors are also essential tools for studying the role of estrogens in adults, or during development [11].

Although a chemical way of synthesis of testolactones is also possible (using an oxidant such as a peroxyacid), the microbial production is much more environmentally friendly.

In the early 1950s Fried [16] and Peterson [17] noted that during the incubation of progesterone with *Aspergillus flavus* and *Penicillium* species, testolactone derivatives were formed, apparently by sequential Baeyer-Villiger oxidation. In one case the product was isolated in 70% yield. There are also other microorganisms that degrade progesterone in a similar manner—to testolactone or 1-dehydrotestolactone. These include *Aspergillus, Cephalosporium, Fusarium, Gliocladium*, and *Streptomyces* species [18].

The androstendione monooxygenase reaction described by Praire and Talaley [19] is a peculiar case of biological oxidation of a steroid: an oxygen atom is inserted into D-ring of the substrate producing a D-homo-17a-oxa-steroid. This enzyme of *Cylindrocarpon radicicola* has been purified and its molecular and catalytic properties characterized [20,21].

In general, the presence of a substituent at 17α-position is a barrier to lactonization by *P. notatum*. Pharmacologically important derivatives of testosterone **1** with 17α-methyl group (17α-methyltestosterone **5** and dianabol **7**) were hydroxylated mainly at 15α-position. Among the microbiological transformations of steroids, 15α-hydroxylation is an industrially important one as it leads to the intermediate for the synthesis of Gestoden [22], a widely used contraceptive drug. Several species of the *Aspergillus, Fusarium, Mortierella* and *Penicillium* are useful with this respect [23].

Reduction of the 4-ene functionality, leading to the formation of the 4,5-dihydroproduct with no further reduction to 3-ol compound was also observed. As we know 15α,17β-dihydroxy-17α-methyl-5α-androstan-3-one **16** is a novel metabolite.

Although the compounds with 17α-methyl group (17α-methyltestosterone **5** and dianabol **7**) were successfully converted to the 15α-hydroxyderivatives, 17α-ethynyltestosterone **8** and noretandrolon **9** were not transformed, the bulk of the substrates being recovered intact. Probably, in both cases considerably large substituents at C-9 were a steric hindrance for the enzyme responsible for introducing the hydroxyl group at 15α position. It is also worth noting that ethynyl-steroids are known as inhibitors of P-450 cytochrome enzymes [24].

The results obtained indicate that the strain of *P. notatum* used in our study is a promising fungus that may be used in commercial processes, especially for production of testolactones. Biotransformations of steroids by this culture proved to be an efficient method of preparation of these biologically important compounds.

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