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Transformations of 4- and 17α-substituted testosterone analogues by *Fusarium culmorum*

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Abstract

A series of 4- and/or 17α-substituted testosterone analogues has been incubated with the hydroxylating fungus *Fusarium culmorum* AM282. It was found that 19-norandrostenedione, 19-nortestosterone, 4-methoxytestosterone, 4-methyltestosterone, and 4-chloro-17α-methyltestosterone were hydroxylated exclusively or mainly at the 6β-position. The mixtures of 6β-, 15α-, and 12β- or 11α-monohydroxy derivatives were obtained from 17α-methyltestosterone and 17α-ethyl-19-nortestosterone—the substrates with alkyl group at C-17α. 4-Chlorotestosterone was predominantly hydroxylated at 15α-position, but the reaction was accompanied by the reduction of 4-en-3-one system, which proceeded in the sequence: reduction of ketone to 3β-alcohol and then reduction of the double 4,5 bond.

The results obtained indicate an influence of stereoelectronic and steric effects of substitutes on regioselectivity of the hydroxylation of 4-en-3-one steroids by *F. culmorum*.

Keywords: Microbial hydroxylation; Anabolic; *Fusarium culmorum*

1. Introduction

A common feature of filamentous fungi is their ability to transform steroids, often by regio- and stereoselective hydroxylation. Although the site of hydroxylation is often a characteristic feature of a fungal genus or species [1,2], some of the collected data indicate that the structure of a substrate can also influence the position of the newly introduced hydroxyl group.

There are reports that fungi belonging to the genus *Fusarium* have been used in steroid transformations [1–9]; however, very little systematic studies on the correlation between the structure of a substrate and the course of hydroxylation have been carried out.

In our previous work, we investigated transformations of the 4-en-3-one and 3β-hydroxy-5-ene steroid hormones and their derivatives by means of *Fusarium culmorum* [10,11]. Although the hydroxylations proceeded with significant regio- and stereospecifities, we observed formation of both mono- and dihydroxy products, with OH groups in different positions of the steroid skeleton. We have noticed that the substrates with 4-ene-3-one group were hydroxylated in one of the following positions: 6β or 15α or 12β, or both 12β and 15α simultaneously. What is more, hydroxylation at 6β took place only in the cause of substrates with an oxygen function at C-17. These results "fit" well to the Brannon and Jones models [12]; therefore, in our opinion *F. culmorum* seems to be a suitable model for studies on correlation between the course of the enzymatic hydroxylation and a substrate structure.

In this paper, we have focused on biotransformations by means of *F. culmorum* of the following substrates: 19-norandrostenedione, 19-nortestosterone, and its 17α-ethyl analogue, and the testosterone derivatives with additional substituents at C-4 or/and C-17.

It is commonly known that structural modifications of steroid hormones influence their biological activity.

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For example, removal of 19-methyl group in testosterone enhances the anabolic to androgenic ratio. Also, high anabolic activity is attributed to the 4-substituted testosterone analogues (especially 4-halogenated compounds). Addition of 17α-methyl group to testosterone halves both the anabolic and androgenic activity but leaving the activity ratio unchanged. 17α-Alkyl substituent protects the C-17 hydroxyl function from oral inactivation. Studies on metabolism of these compounds in human and in other mammals are well documented [13–28], but the structures of the hydroxylated metabolites have not been fully elucidated [22–28], due to the lack of the specific reference standards.

2. Experimental

2.1. Microorganism

The microorganism *F. culmorum* AM282 used in the present study was obtained from the collection of the Institute of Biology and Botany of the Medical University of Wrocław.

2.2. Conditions of cultivation and transformation

The strain of *F. culmorum* was maintained on a Sabouraud 4% dextrose agar slope and was freshly subcultured before using in the biotransformation experiment.

Three hundred milliliters Erlenmeyer flasks, each containing 100 ml of the medium consisting of 3% glucose and 1% peptone, were inoculated with a suspension of *F. culmorum* and then incubated for 3 days at 20 °C in a rotary shaker. After full growth of the microorganisms, 20–25 mg of a substrate in 1 ml of acetone was added to it, and the transformation was continued for a few days under the same conditions. Microbial conversions of substrates were monitored by TLC and GC.

2.3. Isolation and identification of the biotransformation products

All the fermentation media were extracted three times with 15–20 ml of chloroform. Then the solvent was evaporated off and the transformation products in the residues were analyzed by TLC, GC and separated by silica gel column chromatography. TLC analysis was carried out using Merck Kieselgel 60 F_{254} plates with hexane/acetone (2:1 or 3:2, v/v) as eluent. Visualization of the steroids was performed by spraying the plates with a mixture of methanol/concentrated sulphuric acid (1:1, v/v) and heating at 100 °C until the colors developed. GC analysis was performed using a Hewlett-Packard 5890A Series II GC instrument (FID, carrier gas H_2 at flow rate of 2 ml/min), equipped with a HP-5 column (cross-linked 5% Ph–Me–siloxane, 25 m × 0.32 mm × 0.52 μm film thickness) for **1**,**2** (temperature conditions: 220 °C/min, 8 °C/min–270 °C, 2 °C/min–300 °C/2 min), for **6**,**7** (220 °C/min, 6 °C/min–250 °C, 2 °C/min–300 °C/5 min), and for **8** (220 °C/min, 10 °C/min–270 °C, 8 °C/min–300 °C/9 min) or equipped with a HP-1 column (cross-linked methyl siloxane, 25 m × 0.32 mm × 0.52 μm film thickness) for **3**–**5** (200 °C/min, 6 °C/min–270 °C, 10 °C/min–300 °C/2 min). Column chromatography was performed using silica gel and hexane/acetone mixtures with gradually increasing acetone content as eluent. Structures of the biotransformation products were determined by means of ^{1}H NMR (the spectra were recorded on DRX 300 MHz Bruker spectrometer and measured in $CDCl_3$ with TMS as an internal standard).

3. Results

3.1. Biotransformations of steroids with F. culmorum

The following substrates were examined: 19-nortestosterone (**1**), 19-norandrostenedione (**2**), 4-methoxytestosterone (**3**), 4-methyltestosterone (**4**), 4-chlorotestosterone (**5**), 17α-methyltestosterone (**6**), 17α-ethyl-19-nortestosterone (**7**), and 4-chloro-17α-methyltestosterone (**8**). The results are given in Table 1. Only the metabolites the yield of which exceeded 10% of all the products (determined by GC) are listed. The structures of substrates and their metabolites are showed in Fig. 1.

3.2. Identification of metabolites

All the products obtained proved to be secondary alcohols. Position and configuration of the introduced hydroxyl groups were determined by the chemical shifts and the shape of C*H*OH signals in the ^{1}H NMR spectra. Assumed structures were confirmed by comparison of chemical shifts of selected, diagnostically significant signals in the NMR spectra of the substrates and the products and the literature data [25,29–31]. ^{1}H NMR data is shown in Table 2.

3.2.1. 6β-Hydroxy derivatives

In ^{1}H NMR spectra of the metabolites with a hydroxyl group at 6β-position a narrow triplet of 6α-proton, and the downfield shift of 19-H_3 signal (about 0.2 ppm with respect to the substrate) confirmed that a 6β-hydroxyl group had been introduced. Chemical shifts of the 6α-proton in the spectra of metabolites without any additional substituent at C-4: δ = 4.35–4.38 ppm for 6β, 17β-dihydroxy compound (**1a**, **6a**, and **7a**), and δ = 4.42 ppm for 6β-hydroxy-17-oxo compound **2a** were in accordance with those reported by Kirk et al. for 6β-hydroxytestosterone and 6β-hydroxyandrostenedione [31].

6α-H signal of the 4-substituted 6β-hydroxy products (**3a**, **4a**–**4d**, **5a**, and **8a**) was significantly shifted downfield due to the influence of a substituent at C-4. In the case of chlorine as a 4-substituent, the difference of the shifts reached as much as 0.96–0.98 ppm. The analogous change of the chemical shifts was observed for 6β-hydroxy-4-

Table 1
Biotransformation of steroids by *Fusarium culmorum*

Substrate	Incubation time (days)	Metabolite	% (by GC)	R_t (min)
19-Nortestosterone (**1**)	3	6β-Hydroxy-19-nortestosterone (**1a**)	32	13.92
		6β-Hydroxy-19-norandrostenedione (**2a**)	48	13.66
19-Norandrostenedione (**2**)	3	6β-Hydroxy-19-nortestosterone (**1a**)	15	13.92
		6β-Hydroxy-19-norandrostenedione (**2a**)	66	13.66
4-Methoxytestosterone (**3**)	4	6β-Hydroxy-4-methoxyandrostenedione (**3a**)	80	11.98
4-Methyltestosterone (**4**)	2	6β-Hydroxy-4-methylandrostenedione (**4b**)	76	9.80
		6β-Hydroxy-4-methyltestosterone (**4a**)	10	10.09
	10	6β,15α-Dihydroxy-4-methylandrostenedione (**4c**)	32	12.11
		6β,11α-Dihydroxy-4-methylandrostenedione (**4d**)	17	11.74
4-Chlorotestosterone (**5**)	1.5	6β-Hydroxy-4-chloroandrostenedione (**5a**)	10	12.53
		15α-Hydroxy-4-chloroandrostenedione (**5b**)	11	13.28
		3β,15α-Dihydroxy-4-chloro-4-androstene-17-one (**5c**)	22	12.45
		3β,15α-Dihydroxy-4α-chloro-5α-androstan-17-one (**5d**)	39	10.70
17α-Methyltestosterone (**6**)	2	6β-Hydroxy-17α-methyltestosterone (**6a**)	50	22.22
		15α-Hydroxy-17α-methyltestosterone (**6b**)	22	24.13
		12β-Hydroxy-17α-methyltestosterone (**6c**)	22	23.63
17α-Ethyl-19-nortestosterone (**7**)	1	6β-Hydroxy-17α-ethyl-19-nortestosterone (**7a**)	43	20.55
		15α-Hydroxy-17α-ethyl-19-nortestosterone (**7b**)	22	22.65
		11α-Hydroxy-17α-ethyl-19-nortestosterone (**7c**)	12	22.16
4-Chloro-17α-methyltestosterone (**8**)	1	6β-Hydroxy-4-chloro-17α-methyltestosterone (**8a**)	73	15.71
		15α-Hydroxy-4-chloro-17α-methyltestosterone (**8b**)	27	18.15

chloro-1,2-dehydro-17α-methyltestosterone in comparison with 6β-hydroxy-1,2-dehydro-17α-methyltestosterone [25]. The presence of 4-methyl group resulted in the change of 6α-H signal position by 0.64 ppm. The same influence of a methyl group was observed by Azerad and co-workers in the case of hydroxylation of 1,4a-dimethyl-4,4a,5,6,7,8-hexahydro-2(*3H*)-naphthalenone [32]. In the spectrum of **3a** the chemical shift of 6α-H signal was shifted downfield by 0.78 ppm, as a result of deshielding effect of C-4-metoxyl group. The observed effect of the substituents at C-4 on chemical shifts of 6α-H decreased in the following order: $Cl > OCH_3 > CH_3$, and was in accordance with that reported in [33].

Additionally, 6β-hydroxyl group effects the chemical shift of 4-CH_3 or 4-OCH_3 protons, moving it downfield by 0.1 ppm.

3.2.2. 15α-Hydroxy derivatives

The presence of 15α-hydroxyl group was indicated by the signal of 15β-H of the shape of a triplet split into doublet ($W_h > 20$ Hz). Its chemical shift in the spectra of 15α,17β-dihydroxy metabolites (**6b**, **7b**, and **8b**) and the 15α-hydroxy,17-oxo metabolites (**5b**–**5c**) ($\delta = 4.10$–4.13 ppm and 4.41–4.45 ppm, respectively) was in accordance with those reported in literature for 15α-hydroxytestosterone and 15α-hydroxyandrostenedione [31]. In the all of 15α-hydroxy-17-oxo products, the signal of 16β-H was observed at $\delta \approx 3$ ppm (dd, $J_{16\alpha,16\beta} = 19.3$ Hz, $J_{15\beta,16\beta} = 8$ Hz). In the spectrum of 6β,15α-dihydroxy product **4c**, in the presence of the additional hydroxyl group at C-6, the signal of 15β-proton was moved by 0.09 ppm.

3.2.3. 6β,11α-Dihydroxy-4-methylandrostenedione (*4d*)

11α-Hydroxylation was characterized by the broad 11β-H signal at $\delta = 4.1$ ppm, and the exceptionally low-field 1β-H signal at $\delta = 2.7$ ppm, which was shifted due to the spatial proximity of 11α-OH. Also, the above-mentioned features indicating 6β-hydroxylation were present. Changes in the chemical shift of 19-H_3 with respect to 6β-hydroxy-4-methylandrostenedione (**4a**) (+0.14 ppm) confirmed the presence of 11α-hydroxyl group.

3.2.4. 3β,15α-Dihydroxy-4-chloro-4-androsten-17-one (*5c*)

The ^{1}H NMR spectrum of **5c** showed two broad C*H*OH signals, which are typical for axial protons. 15α-Hydroxylation was indicated by spectral similarities to other 15α-alcohols. Reduction of the 3-keto group was recognized by up-field shift of 19-H_3 signal with respect to the 15α-hydroxy-4-chloroandrostenedione (**5b**) (−0.2 ppm, which is in accordance with the literature [34]).

3.2.5. 3β,15α-Dihydroxy-4α-chloro-5α-androstan-17-one (*5d*)

In the ^{1}H NMR spectrum of **5d** all the characteristic features associated with 15α-hydroxylation of 17-keto compounds (see above) were present. Significant up-field shift of 19-H_3 singlet indicated that the 4,5 double bond was reduced.

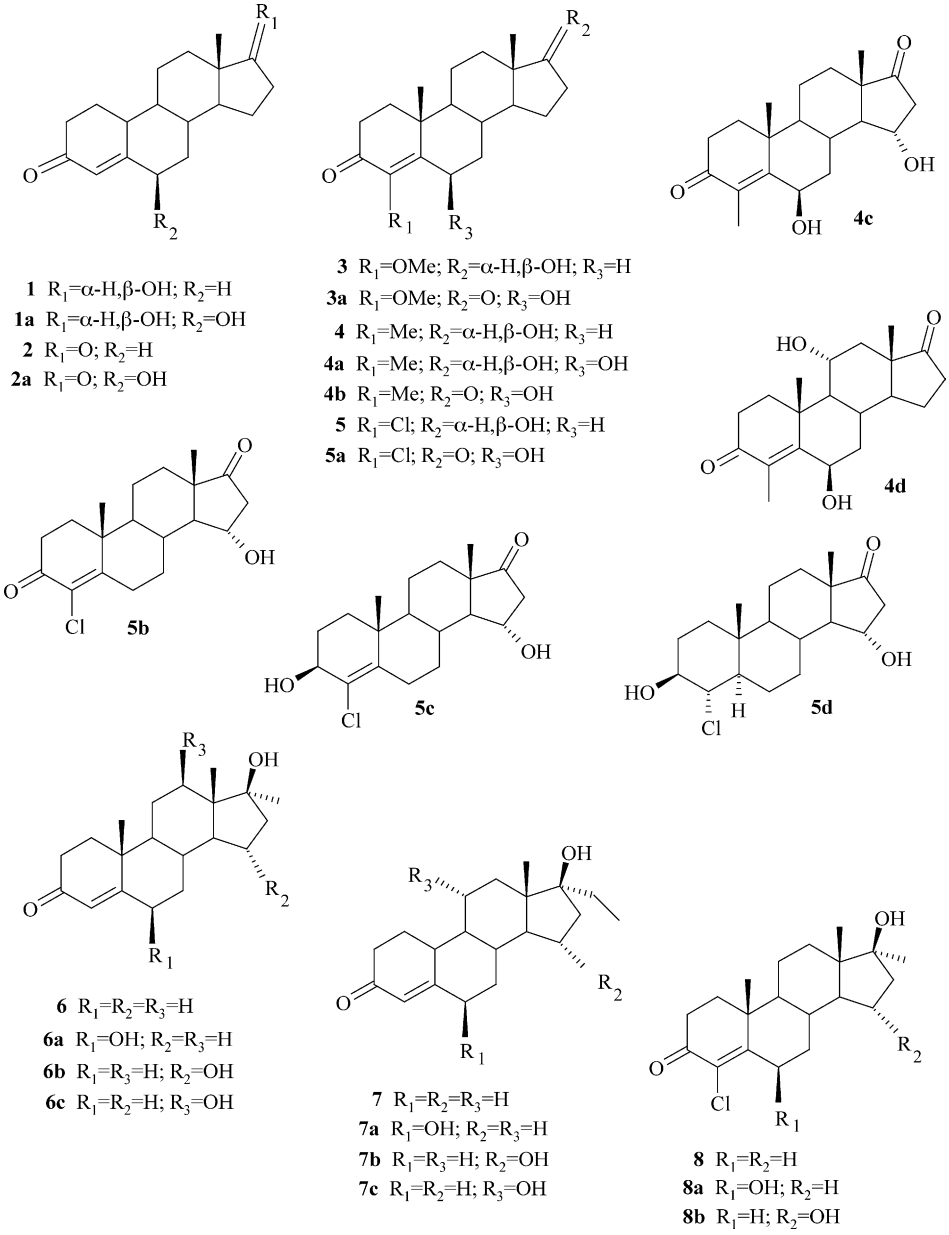

Fig. 1. Structures of substrates and products of their transformations.

A special feature of the spectrum were the two broad signals of axial protons: a triplet at $\delta = 3.76$ ppm (4β-H) and a characteristic multiplet of 3α-H at $\delta = 3.55$ ppm. These signals could be identified as 4α-H and 3β-H in 5β-series, or 4β-H and 3α-H in 5α-series, respectively. The assignment to the 5α-H configuration was supported by the lower chemical shift of the H-4 triplet compared to the one observed for 5β-series [35].

*3.2.6. 12β-Hydroxy-17α-methyltestosterone (**6c**)*

The location of a hydroxyl group at the 12β-position was proved by the shape of the C*H*OH signal (the axial proton is coupled with two other protons only), and a downfield shift of 17α-methyl protons. The chemical shifts of the significant signals in ^{1}H NMR of this metabolite are consistent with the literature data [29].

*3.2.7. 11α-Hydroxy-17α-ethyl-19-nortestosterone (**7c**)*

The shape of the multiplet at $\delta = 3.91$ ppm (triplet split into doublet) is similar to that of 11β-H in ^{1}H NMR spectrum of 6β,11α-dihydroxy-4-methylandrostenedione (**4d**). Chemical shift of 18-H$_3$ group is in accordance with the one calculated from increments given by Kirk [31].

4. Discussion

Our previous research showed that the strain *F. culmorum* AM282 is capable of effective regioselective hydroxylation of 4-en-3-one steroid hormones (testosterone, androstenedione, progesterone) [10]. We also showed that the hydroxylations of these hormones at 6β-, 12β-, and 15α-positions

Table 2
Chemical shifts of the substrates and their metabolites in ^{1}H NMR spectra (δ ppm, J Hz; in $CDCl_3$)

Compound	4-H	17α-H	18-H_3	19-H_3	C*H*OH	Other significant signals	Remarks
19-Nortestosterone (**1**)	5.82s	3.66t (J = 8.4)	0.81s	–	–	–	
19-Norandrostenedione (**2**)	5.82s	–	0.95s	–	–	–	
6β-Hydroxy-19-nortestosterone (**1a**)	5.88s [5.88]	3.66t (J = 8.5) [3.65t, J = 8.1]	0.82 [0.81]	–	4.38t (J = 2.5) (6α-H) [4.37t, J = 2.5]	–	[29]
6β-Hydroxy-19-norandrostenedione (**2a**)	5.89s	–	0.94s	–	4.42t (J = 2.5) (6α-H)	–	
4-Methoxytestosterone (**3**)	–	3.57t (J = 9.0)	0.77s	1.18s	–	3.57brs (4-OCH_3)	
6β-Hydroxy-4-methoxyandrostenedione (**3a**)	–	–	0.94s	1.40s	5.19t (J = 2.7) (6α-H)	3.66brs (4-OCH_3)	
4-Methyltestosterone (**4**)	–	3.65t (J = 9.0)	0.79s	1.18s	–	1.78s (4-CH_3)	
6β-Hydroxy-4-methylandrostenedione (**4b**)	–	–	0.95s	1.40s	5.05t (J = 2.9) (6α-H)	1.89s (4-CH_3)	
6β-Hydroxy-4-methyltestosterone (**4a**)	–	3.65t (J = 9.0)	0.83s	1.38s	5.00t (J = 2.9) (6α-H)	1.87s (4-CH_3)	
6β,15α-Dihydroxy-4-methyl-androstenedione (**4c**)	–	–	0.98s	1.45s	5.05t (J = 3.1) (6α-H); 4.50m (W_h = 23.0 Hz) (15β-H)	1.85s (4-CH_3); 3.00dd (J = 19.3; J = 8.0) (16β-H)	
6β,11α-Dihydroxy-4-methyl-androstenedione (**4d**)	–	–	0.98s	1.55s	5.10t (J = 3.0) (6α-H); 4.10dt (J = 11.0; J = 4.5) (11β-H)	1.90s (4-CH_3); 2.70dt (J = 14.5; J = 4.5) (1β-H)	
4-Chlorotestosterone (**5**)	–	3.66t (J = 8.0)	0.80s	1.24s	–	–	
6β-Hydroxy-4-chloro-androstenedione (**5a**)	–	–	0.98s	1.45s	5.36t (J = 3.0) (6α-H)	–	
15α-Hydroxy-4-chloro-androstenedione (**5b**)	–	–	0.96s	1.27	4.41m (W_h = 23.5 Hz) (15β-H)	2.99dd (J = 19.3; J = 8.0) (16β-H)	
3β,15α-Dihydroxy-4-chloro-4-androstene-17-one (**5c**)	–	–	0.93s	1.07s	4.40m (W_h = 16.0 Hz) (3α-H); 4.45m (W_h = 24 Hz) (15β-H)	2.97dd (J = 19.3; J = 8.0) (16β-H)	
3β,15α-Dihydroxy-4α-chloro-5α-androstan-17-one (**5d**)	3.76t (J = 10.0)	–	0.90s	0.89s	3.55m (W_h = 23.0 Hz) (3α-H); 4.41m (W_h = 23 Hz) (15β-H)	2.99dd (J = 19.3; J = 8.0) (16β-H)	
17α-Methyltestosterone (**6**)	5.72s	–	0.91s	1.21s	–	1.21s (17α-CH_3)	
6β-Hydroxy–17α-methyltestosterone (**6a**)	5.82s [5.82]	–	0.93s [0.94]	1.40s [1.40]	4.35t (J = 2.9) (6α-H) [4.36t, J = 2.8]	1.22s (17α-CH_3) [1.22]	[25]
15α-Hydroxy–17α-methyltestosterone (**6b**)	5.73s	–	0.92s [0.91]	1.21s [1.21]	4.13m (W_h = 20 Hz) (15β-H)	1.35s (17α-CH_3) [1.34]	[30]
12β-Hydroxy-17α-methyltestosterone (**6c**)	5.73s [5.72]	–	0.97s [0.96]	1.21s [1.20]	3.76dd (J = 10.8; J = 4.6) (12α-H) [3.75dd, J = 11; J = 4.8]	1.35s (17α-CH_3) [1.34]	[29]
17α-Ethyl-19-nortestosterone (**7**)	5.81s	–	0.93s	-	–	0.97t (J = 7.2) (17α-CH_2CH_3)	
6β-Hydroxy-17α-ethyl-19-nortestosterone (**7a**)	5.88s	–	0.96s	-	4.37t (J = 2.8) (6α-H)	1.07t (J = 7.2) (17α-CH_2CH_3)	
15α-Hydroxy-17α-ethyl-19-nortestosterone (**7b**)	5.82	–	0.95s	-	4.12m (W_h = 22.2 Hz) (15β-H)	1.01t (J = 7.2) (17α-CH_2CH_3)	
11α-Hydroxy-17α-ethyl-19-nortestosterone (**7c**)	5.81s	–	0.95s	-	3.91dt (J = 10.0; J = 4.5) (11β-H)	0.98t (J = 7.2) (17α-CH_2CH_3)	
4-Chloro-17α-methyltestosterone (**8**)	–	–	0.91s	1.24s	–	1.21s (17α-CH_3)	
6β-Hydroxy-4-chloro-17α-methyltestosterone (**8a**)	–	–	0.93s	1.42s	5.33t (J = 3.0) (6α-H)	1.21s (17α-CH_3)	
15α-Hydroxy-4-chloro-17α-methyltestosterone (**8b**)	–	–	0.92s [0.94]	1.26s [1.27]	4.10m (W_h = 21.5 Hz) (15β-H)	1.35s (17α-CH_3) [1.37]	[30]

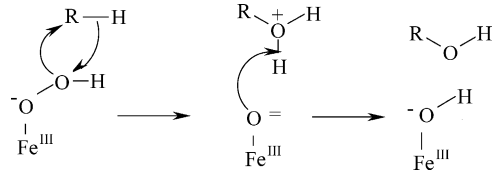

Fig. 2. Mechanism of hydroxylation by means of P450 hydroperoxo-iron species involving insertion of OH^+.

were carried out by the same enzyme complex, and that a nature of a substituent at C-17 had a significant effect on the regioselectivity of the reaction.

Continuing our research on relationship between a substrate structure and a hydroxylation profile we carried out the transformation of the following substrates: 19-nortestosterone, 19-norandrostenedione and C-4 and/or C-17 substituted testosterone analogues. Some of the substrates (19-nortestosterone (**1**), 19-norandrostendione (**2**), 4-metoxytestosterone (**3**), and 4-methyltestosterone (**4**)) underwent selective 6β-hydroxylation, whereas 4-chlorotestosterone (**5**) gave mainly 15α-hydroxy derivatives (in above 70% yield) (Table 1). Time course experiments evidently indicated (data not presented) that the first stage of the transformation was an oxidation of the hydroxyl group at C-17, which means that the isolated products were formed from 17-oxo substrates. The results obtained showed that both steric and stereoelectronic factors have an impact on the regioselectivity of hydroxylations by means of *F. culmorum*. They are consistent with results obtained for other microorganisms [5,36].

The 19-nor-substrates (**1**,**2**) underwent regioselective 6β-hydroxylation, whereas the transformation of androstenedione, carried out under the same conditions, gave 6β- and 15α-hydroxy derivatives (ratio 1:2, respectively) [10]. Lack of a methyl group in the 1-3 *cis* position promoted stereoelectronically favoured 6β-hydroxylation.

It might be expected that the presence of a substituent at C-4 would prevent the hydroxylation at the relatively close 6β-position, due to the steric hindrance. It was then highly surprising to find that the 6β-hydroxy derivatives were the only products of the transformations of 4-methyltestosterone (**4**) and 4-metoxytestosterone (**3**). Also, a 6β-hydroxy compound was the major product of the biotransformation of one of the 17α-alkyl substrates: 4-chloro-17α-methyltestosterone (**8**).

The results presented above indicate that the influence of a substituent at C-4 on the reaction of enzymatic hydroxylation is similar to the one observed in electrophilic substitution in aromatic systems. This support the mechanism proposed by Newcomb et al. [37], which assumes a transfer of OH^+ cation from hydroperoxo-iron species P450 to the substrate molecule (Fig. 2). An electrodonating or/and possessing free valence electrons substituent at C-4 promotes (by means of π-electrons) attack of OH^+ and decreases electrodeficient properties of the intermediate oxo-cation. As

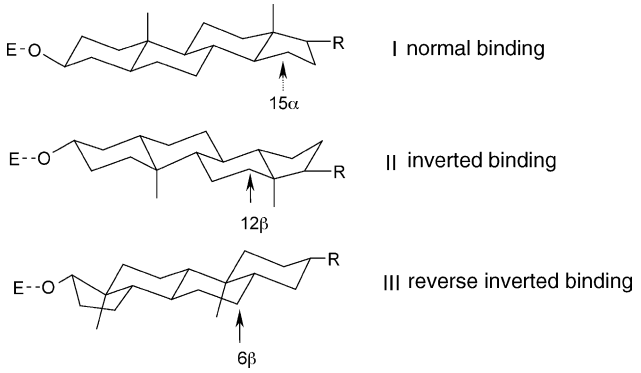

Fig. 3. Enzyme–substrate complexes with different orientation of steroid molecules.

a result, the hydroxylation occurs in the allylic, axial 6β-position.

The biotransformations of the 17α-alkyl derivatives: 17α-methyltestosterone (**6**) and 17α-ethyl-19-nortestosterone (**7**) gave mixtures of 6β-, 15α- and, respectively, 12β- or 11α-alcohols. The mixtures contained less 6β-allyl alcohol than in the case of the transformation of 17α-unsubstituted testosterone. Monitoring of the progress of the testosterone biotransformation (by means of GC) showed that testosterone initially undergoes 6β-hydroxylation [10]—no other hydroxy derivatives were detected in the reaction after 12 h of incubation. The lower contents of 6β-hydroxy products in the transformations of the 17α-alkyl compounds may arise from steric factors associated with the 17α-substituent. These may effect formation of the enzyme–substrate complex with participation of the 17β-hydroxyl group (III, Fig. 3). At the same time, the 17α-substituent is a drawback for 1-3 *cis* hydroxylation at 15α-position, therefore the hydroxylation at the equivalent 12β-position occurs (II, Fig. 3).

Similar effect of substituents on the regioselectivity of the hydroxylation was observed in transformations of progesterone and 17α-acetoxy-progesterone by the strain *Calonectria decora* [38]. Progesteron was hydroxylated at 12β,15α-positions, whereas 17α-acetoxy-progesterone only at 12β-position.

The 11α-hydroxy derivative of 17α-ethyl-19-nortestosterone (**7**) is probably formed via the complex which usually leads to 12β-alcohol (II, Fig. 3), but in the presence of the bulkier ethyl group (compared to the methyl one) the more distant 11α-position is hydroxylated. The different stereochemistry of the newly introduced hydroxyl group (11α- instead of 12β-) may arise from the general tendency of the strain to hydroxylation of equatorial C–H bonds.

4-Chlorotestosterone (**5**) was transformed in a different way: apart from the hydroxylation and the oxidation of C-17 hydroxyl group, the reduction of 4-en-3-one system in the A-ring was observed. The first step of this reduction was formation of the 3β-allyl alcohol (Fig. 4). Microbial reduction of an α,β-unsaturated ketone to an allyl alco-

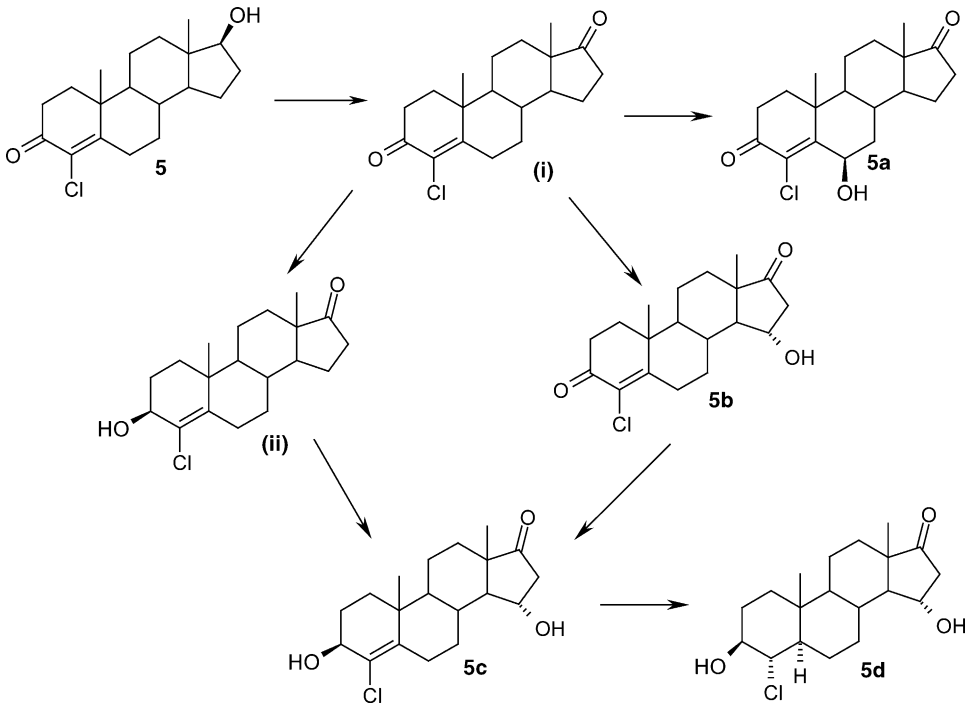

Fig. 4. Proposed metabolic pathway of 4-chlorotestosterone by *F. culmorum*. (i) and (ii) were isolated only after 12 or 24 h of transformation.

hol is a rarely observed process [39,40]. Much more often, there are α-chlorosubstituted α,β-unsaturated ketones that undergo this type of transformation. This is because the presence of the chlorine atom decreases unsaturated character of a conjugate ketone [41]. The fact that 4-chloro-17α-methyltestosterone does not undergo the reduction in the A ring indicates that the presence of the 17-oxo group has an impact on this reaction. The products of the transformation of 4-chlorotestosterone were mainly the 15α-hydroxy derivatives. 4-Chloroandrostendione (i) and 3β-hydroxy-4-chloro-4-androsten-17-one (ii) (Fig. 4) were identified in the reaction mixture after 12 and 24 h of the transformation, but they were absent in the final mixture (data not presented). This suggest that they also underwent the C-15 hydroxylation.

The course of transformations of the tested steroids by *F. culmorum* indicated that the 6β-hydroxy metabolism played minor role when the metabolism of the A-ring was rapid, as it was in the case of 4-chlorotestosterone (**5**). Similar dependence was observed in the metabolism of anabolic steroids in humans [25].

In general, the biotransformations of the tested substrates led mainly to monohydroxy products. Only 4-methyltestosterone (**4**), when subjected to the reaction for longer time (10 days), gave 6β,11α- and 6β,15α-dihydroxy derivatives. In our earlier works with *F. culmorum* we also observed formation of dihydroxy products in the case of other relatively lipofilic substrates [10,11].

Twelve of the metabolites obtained are the new compounds. The hydroxy derivatives isolated from the biotransformation of 4-chlorotestosterone may be helpful in tracing the fate of this anabolic steroid in mammals.

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