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Biotransformation XLV. Transformations of 4-Ene-3-oxo Steroids In *Fusarium Culmorum* Culture

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The course of transformations of five 4-ene-3-oxo steroids with varying substituents at C-17 i.e.: 4-androsten-3-one, androstenedione, testosterone, progesterone and 17α-hydroxyprogesterone in *Fusarium culmorum* culture was investigated. All the substrates were hydroxylated either at 12β and 15α, or at 15α or 6β positions, depending on the structure of the substrate. The main product of 4-androsten-3-one transformation was 12β,15α-diol. A similar 12β,15α-diol was obtained from progesterone, but the main product of transformation of this substrate was 15α-hydroxyprogesterone. The products of hydroxylation at 6β or 15α positions were isolated from 17α-hydroxyprogesterone. The androstenedione and testosterone transformation mixtures contained the same products (6β-hydroxyandrostenedione, 6β-hydroxytestosterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone), but the quantities of 6β- and 15α-alcohols varied, depending on the substrate used. During transformations of these two substrates, apart from hydroxylation, ketone–alcohol interconversion at C-17 occurred. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The hydroxylation of steroids is important because of its physiological role in mammalian organisms and also for practical reasons [1, 2]. Recognition of transformations in the culture of some microorganisms of steroid hormones and their derivatives, especially those used as drugs, can provide useful information about metabolic processes of xenobiotics in mammals because the metabolic tract in mammals and microorganisms is often similar [3].

The present study was focused on the course of hydroxylation of 4-ene-3-oxo steroids in the culture of *Fusarium culmorum*.

The literature data indicate the ability of fungi of the genus *Fusarium* to hydroxylate on steroidal substrates [4, 5], e.g. some *Fusarium* strains are good hydroxylators at the 11α position of Reichstein's substance S [6, 7]. The 15α hydroxyl group was introduced to hormones: testosterone, androstenedione, progesterone and estrone by all the *Fusarium* strains able to hydroxylate these substrates [4, 5, 8–11]. Quite a large number of these strains converted androstenedione and testosterone to a mixture of 6β- and 15α-hydroxyderivatives. (Čapek [8] found that 92 among 98 investigated strains of *Fusarium* hydroxylated androstenedione and testosterone at 6β and 15α sites.) The strain *F. moniliforme* affected 15α-hydroxylation of estrone and estradiol and 6β-hydroxylation of estradiol 3-methyl ether [9]. 15α hydroxylation of 3β-hydroxy-5α-androst-17-one was observed in transformations by *F. graminearum* whereas 3β-hydroxy-5-androsten-17-one was mostly hydroxylated by this same strain at the 7α-site [12]. The products of hydroxylation at 15α position were formed in testosterone, androstenedione and progesterone transformations by *F. lini*, but only the progesterone was converted to 12β,15α-diol [10].

The presented data indicate that the hydroxylation at 15α position of steroidal skeleton by fungi of the genus *Fusarium* is most frequent, and the structure of the substrate can influence the position of the introduced hydroxyl group. However there are no reports in literature on the relationship between the structure of the substrate and the course of hydroxylation by *Fusaria*. For this reason transformations of five 4-ene-3-oxosteroids, varying in substituents at C-17, were carried out by *F. culmorum*. We have expected that the chosen strain, known for its ability to carry out

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efficient, regioselective hydroxylation of terpene lactone [13], will also be able to carry out effective and selective hydroxylation of steroidal substrates. The purpose of the experiments was to find the differences in the course of transformations of the substrates under investigation. We expected that the comparison of time-course of testosterone and androstenedione transformations would allow to determine the influence of the presence carbonyl and β-hydroxyl group at C-17, although ketone–alcohol interconversion at C-17 was often observed during transformation by *Fusaria* [8, 10].

MATERIALS AND METHODS

Microorganism

F. culmorum was obtained from the collection of the Institute of Biology and Botany, Medical University, Wrocław.

Conditions of cultivation and transformation

(A) The fungi were incubated on 3% glucose and 1% peptone, and shaken at 20°C in 300 ml Erlenmeyer flasks with 80 ml of medium. After three days of growth, the substrate (20 mg) dissolved in 1 ml of acetone was added. The products were extracted with chloroform after 12, 24, or 36 h.

(B) A three-day-old culture (prepared as described in A) was supplemented with 1 mg of androstenolone (3β-hydroxy-5-androsten-17-one), and 20 mg of androstenedione was added after 8 h. The products were extracted with chloroform 12 h later.

Product analysis

The composition of crude mixtures was analyzed by TLC and GLC. TLC was carried out using Silicagel 60 plates with hexane–acetone (2:1 or 1:1 v/v) as eluents. Steroids were detected on plates with H_2SO_4–EtOH (1:1 v/v).

GLC analysis was performed using a Hewlett Packard 5890 Series II gas chromatograph; HP-5 (cross-linked 5% Ph–Me–Silicone) 30 m/0.53 mm/ 0.88 μm film thickness (column 240° min^{-1}, 5–300°C min^{-1}).

The products were separated chromatographically on silica gel 0.05–0.2 (Merck) with hexane–acetone (2:1 v/v) mixture as eluent.

^{1}HMR spectra were obtained in $CDCl_3$ using a Bruker Avance DRX 300, 300 MHz spectrometer.

RESULTS

The transformations were carried out for 36 h. The yield of products was determined using GLC analysis of the crude mixture. The structure of the products after separation in column chromatography was assigned based on ^{1}H-NMR spectra.

Spectra

*Transformation of 4-androsten-3-one (**1**).* 7% of 15α-hydroxyandrostenedione (**6**), 38% of 12β,15α-dihydroxy-4-androsten-3-one (**7**) and 40% of unreacted substrate (**1**) were found in the mixture after transformation of **1**.

*Transformation of testosterone (**2**) and androstenedione (**3**).* GLC analysis of crude mixtures after transformation of substrates **2** and **3** indicated the presence of the same compounds: 6β-hydroxyandrostenedione (**9**), 6β-hydroxytestosterone (**8**), 15α-hydroxyandrostenedione (**6**) and 15α-hydroxytestosterone (**10**), but percentage composition of those alcohols varied depending of the substrate used. Table 1 shows the yield of products formed after 12, 24 and 36 h transformations of **2** and **3** and composition of products mixture formed from androstenedione in *F. culmorum* culture with androstenolone used as inductor.

*Transformation of progesterone (**4**).* Transformation of progesterone (**4**) yielded a mixture of 47% of 15α-hydroxyprogesterone (**11**), 25% of 12β,15α-dihydroxyprogesterone (**12**), and 22% of unreacted substrate **4**.

*Transformation of 17α-hydroxyprogesterone (**5**).* Transformation of 17α-hydroxyprogesterone (**5**) gave a mixture of many products, some of them less polar than the substrate. Only two more polar compounds: 7% of 6β,17α-dihydroxyprogesterone (**13**), and 11% of 15α,17α-dihydroxyprogesterone (**14**) were identified.

Identification of metabolites

The position and configuration of the introduced hydroxyl groups were recognized mainly from chemical shift and shape of proton signals of C*H*OH [14]. The presence of signals for 4-H (5.7–5.9 ppm), for 18-H (0,7–1 ppm) and for 19-H (1,2–1,4 ppm) in ^{1}H-NMR spectra showed that the products had retained their fundamental 4-ene-3-one steroid skeleton (Table 2). The values of chemical shifts of significant signals of products **6**, **8**, **9**, **10**, **11**, and **12** were in agreement with those reported in literature [15].

*6β-Hydroxyandrostenedione (**9**).* The presence of triplet (J = 2,8 Hz) at δ = 4.42 ppm and downfield shift: 0.08 ppm for 4-H, 0.023 ppm for 18-H_3 and 0.20 ppm for 19-H_3 confirmed the presence of 6β-hydroxyl group.

*6β-Hydroxytestosterone (**8**).* Triplet at δ = 3.67 was similar in shape to 17α proton in testosterone spectrum. The shape of signal at δ = 4.36 and changes in signal position (0.11 ppm for 4-H, 0.026 ppm for 18-H_3 and 0.2 ppm for 19-H_3 as compared with testosterone) were similar to those observed in **9**.

*15α-Hydroxyandrostenedione (**6**).* The presence of 15α-hydroxyl group was indicated by the signal at δ = 4.42 ppm (in shape of a triplet split into doublet

Table 1. Composition of crude mixture obtained in androstenedione and testosterone transformations

Compounds present in mixture (%)	Time of incubation, substrate						
	12 h			24 h		36 h	
	testosterone	androstenedione	androstenedione[a]	testosterone	androstenedione	testosterone	androstenedione
Androstenedione (**3**)	25	65	33	15	2	–	–
Testosterone (**2**)	60	6	4	3	–	–	–
6β-*H*ydroxyandrostenedione (**9**)	2	7	17	30	25	32	20
6β-Hydroxytestosterone (**8**)	8	1	2	12	4	10	6
15α-*H*ydroxyandrostenedione (**6**)	3	14	33	28	35	18	21
15α-*H*ydroxytestosterone (**10**)	–	–	3	10	30	22	32

[a]Transformation by culture induced with androstenolone.

Table 2. ^{1}H NMR data for products of transformation by Fusarium culmorum

Compound	4-H	18-H_3	19-H_3	21-H_3	C*H*OH	C*H*OH	Other significant signals
6β-Hydroxyandrostenedione (**9**)	5.84	0.94	1.43	–	4.42t, J = 2,8 Hz(6α-H)	–	
6β-Hydroxytestosterone (**8**)	5.84	0.83	1.41	–	4.36t, J = 2,6 Hz (6α-H)	3.67t, J = 8,2 Hz (17α-*H*)	
6β,17α-Dihydroxyprogesterone (**13**)	5.83 (5.84)[a]	0.81 (0.80)	1.39 (1.39)	2.29 (2.29)	4.37t, J = 2,7 Hz(6α-H)	–	
15α-Hydroxyandrostenedione (**6**)	5.74	0.96	1.23	–	4.42 m, W_h=22 Hz (15β-H)	–	3.07dd, J = 18 Hz, 8 Hz (16β-*H*)
15α-Hydroxytestosterone (**10**)	5.74	0.82	1.22	–	4.11 m, W_h=21 Hz (15β-H)	3.9t, J = 8,4 Hz (17α-H)	
15α-Hydroxyprogesterone (**11**)	5.74	0.69	1.20	2.14	4.11 m, W_h=22 Hz (15β-H)	–	
12β,15α-Dihydroxyprogesterone (**12**)	5.75	0.80	1.20	2.15	4.24 m, W_h=20 Hz (15β-H)	3.55dd, J = 10 Hz, 5 Hz (12α-H)	2,8t, J = 8,5 Hz (17α-*H*)
12β,15α-Dihydroxy-4-androsten-3-one (**7**)	5.78	0.82	1.22	–	4.25 m, W_h=20 Hz (15β-H)	3.55dd, J = 10 Hz, 5 Hz (12α-H)	
15α,17α-Dihydroxyprogesterone (**14**)	5.75 (5.76)	0.775 (0.8)	1.206 (1.19)	2.26 (2.30)	4.15 m, W_h=20 Hz (15β-H)	–	3.26dd, J = 16 Hz, 10 Hz (16β-H)

[a]In parentheses are given chemical shifts calculated from data of 17α-hydroxyprogesterone and increments for 15α- or 6β-hydroxyl group.

— characteristic of 15β proton) and by presence of the signal of 16β proton at $\delta = 3.07$ [dd $J_{16\alpha,16\beta}$=18 Hz and $J_{15\beta,16\beta}$=8 Hz].

15α-Hydroxytestosterone (**10**). Signal at $\delta = 4.11$ similar to that of 15β proton in 15α-hydroxyandrostenedione (**6**) and position of triplet of 17α proton at $\delta = 3.9$ ppm shifted 0.25 ppm downfield, as compared with testosterone, confirmed the presence of 15α-hydroxyl group.

15α-Hydroxyprogesterone (**11**). Chemical shifts of 4-H and 19-H_3 signals in spectrum of **11** were the same as those of progesterone. Signal at $\delta = 4.11$ was similar in shape to that of 15β hydrogen signal in 15α-hydroxytestosterone spectrum. Changes in signal position for 18-H_3 (0.03 ppm) and for 21-H_3 (0.015 ppm) confirmed the presence of 15α-hydroxyl group.

12β,15α-Dihydroxyprogesterone (**12**). Signals at $\delta = 4.24$ and $\delta = 3.55$ indicated the presence of two hydroxyl groups. Similarity of signal at $\delta = 4.24$ to 15β proton signal of 15α-hydroxyprogesterone (**11**) showed on the presence of 15α-hydroxyl group. The shape of the signal at $\delta = 3.55$ suggests that it belongs to axial proton coupled with only two protons, therefore it can be either the signal of 1α or 12α proton. Chemical shift of this signal and 0.1 ppm shift in signal position of 15β proton as compared with 15α-alcohol **11** confirmed the presence of 12β hydroxyl group. Positions of signals of angular methyl groups were in agreement with those reported for **11** [15].

12β,15α-Dihydroxy-4-androsten-3-one (**7**). Position and shape of C*H*OH signals were similar to those present in the spectrum of 12β,15α-dihydroxyprogesterone (**12**). Positions of signal of 18-H_3 and 19-H_3 were in agreement with those calculated for **7** [16].

6β,17α-Dihydroxyprogesterone (**13**). The narrow profile of signal at $\delta = 4.37$ and 0.08 ppm downfield shift for 4-H confirmed the presence of 6β-hydroxyl group. The downfield shifts in signals for 18-H_3 (0.03 ppm) and 19-H_3 (0.21 ppm) were the same as those observed for 6β-alcohols **8** and **9**.

15α,17α-Dihydroxyprogesterone (**14**). The shape of signal at $\delta = 4.15$ ppm similar to that of 15α-alcohols **6** and **11**, and signal of 16β proton at $\delta = 3.26$ [dd $J_{16\alpha,16\beta}$=16 Hz, $J_{15\beta,16\beta}$=10 Hz] confirmed the presence of 15α-hydroxyl group. The change in signal position as compared with that observed for substrate **5** for 19-H_3 (0.016 ppm) was in agreement with the data observed earlier for 15α alcohols **6**, **10** and **11**, but 0.005 ppm (instead of 0.03 ppm) for 18-H_3 was obtained unexpectedly. Similarly, the change in signal position for 18-H_3, lower than normal for 15α-hydroxyderivatives, was observed when 15α-hydroxyl group was introduced to 17α-methyltestosterone [3]. The position of signals: 18-H_3, 19-H_3, 21-H_3 methyl groups and 4-H proton is in agreement to reported in literature [13] for chemically synthesized compound **14**.

DISCUSSION

The course of transformations of five 4-ene-3-oxo steroids with varying substituents at C-17, i.e.: 4-androsten-3-one, testosterone, androstenedione, progesterone and 17α-hydroxyprogesterone in *F. culmorum* culture was investigated (Fig. 1).

All the substrates were efficiently hydroxylated. The hydroxylation occurred either at 12β and 15α or at 6β or 15α sites, depending on the substrate. Small amounts of product oxidation at C-17 (15α-hydroxyandrostenedione) were also obtained from 4-androsten-3-one — the substrate with no substituent at C-17. But introduction of two equatorial 12β and 15α hydroxyl groups was the main reaction that occurred during transformation of this substrate. An analogous 12β,15α-diol **12** was produced during progesterone transformation, but the main product obtained from progesterone was 15α-alcohol **11**.

Testosterone (**2**) and androstenedione (**3**) transformations yielded the same products: 6β-hydroxyandrostenedione, 6β-hydroxytestosterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone, but the percentage of alcohols varied, depending on the substrate used. During transformations of substrates **2** and **3**, apart from hydroxylation, ketone–alcohol interconversion at C-17 occurred. The ketone–alcohol interconversion at C-17 made, that both androstenedione and testosterone were present in the culture, irrespective of the substrate (**2** or **3**) added. The differences in the ratio of 6β- and 15α-alcohols obtained from **2** and **3** indicate, that the presence of carbonyl or 17β-hydroxyl group influenced the course of hydroxylation by *F. culmorum*. To estimate the yield of 6β- and 15α-hydroxyproducts formed in the transformations of each substrate, we studied the time-course of their transformations. We compared the composition of products obtained from **2** and **3** after transformations carried out for: 12, 24 and 36 h. The differences in quantitative composition of the products obtained from androstenedione and testosterone transformations carried out for different time (Table 1) allowed us to determine the amounts of 6β- and 15α-alcohols produced in the transformations of each substrate.

Testosterone (**2**) and androstenedione (**3**) transformations carried out parallelly show that dione **3** was hydroxylated faster than keto-alcohol **2**; after 12 h of incubation of **2** and **3** 13% and 22% of all products of hydroxylation were formed respectively (Table 1). Small amounts of **2**, about ten times smaller than those of **3**, present in the product mixture of **3** and faster transformation **3** than **2** indicate that the products obtained from **3** are the results of its hydroxylation. The composition of androstenedione transformation mixture shows that in the presence of the carbonyl group at C-17, hydroxylation at 6β and 15α sites occurred and the total amount of 15α-alco-

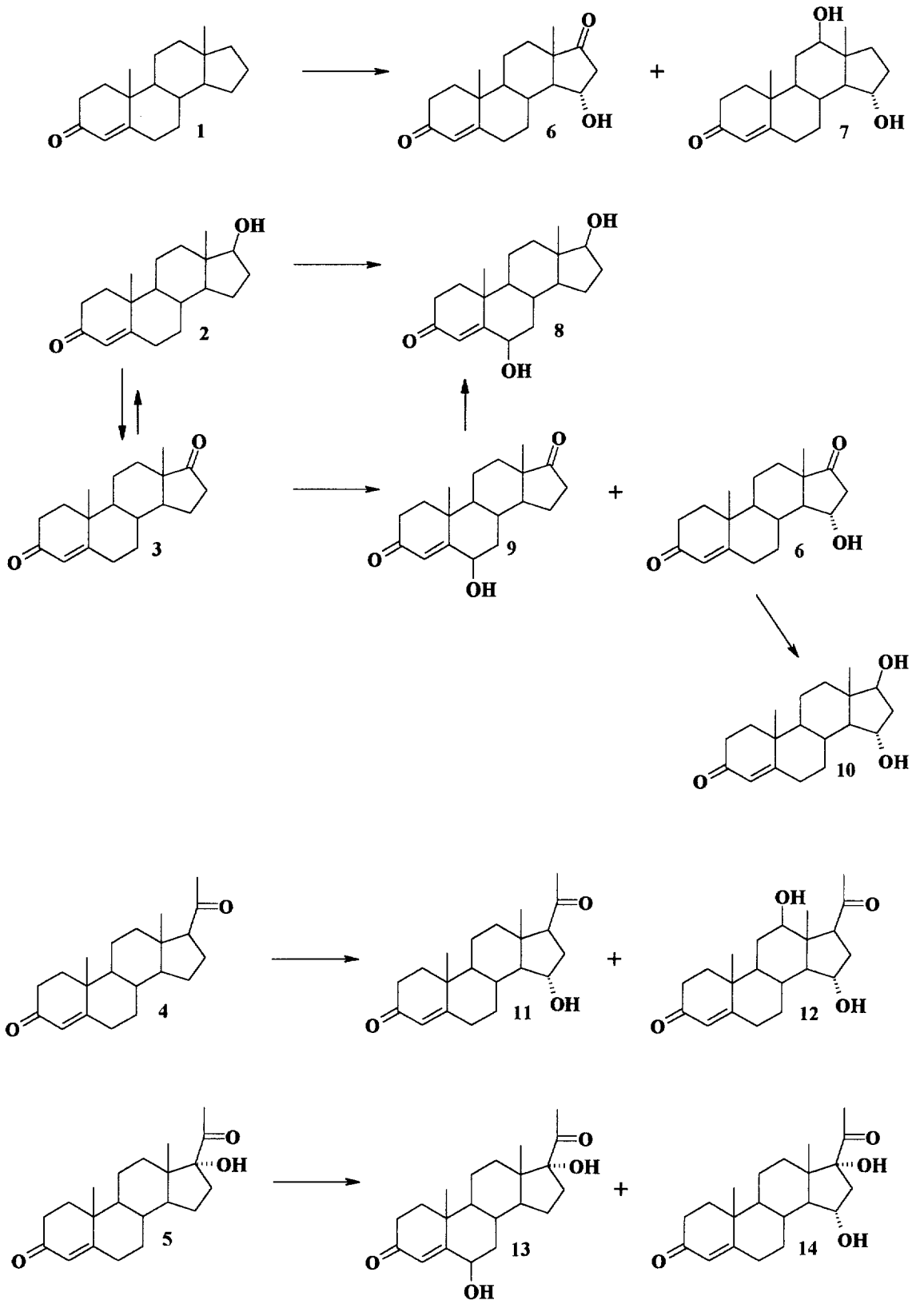

Fig. 1. Metabolism of 4-ene-3-oxo steroids by *Fusarium culmorum*

hols **6** and **10** was about twice that of 6β-hydroxyderivatives **8** and **9**. Reduction of the carbonyl group at C-17 to β-alcohol occurred in the two products of androstenedione hydroxylation, but 15α-hydroxyandrostenedione was reduced more efficiently than 6β-hydroxyandrostenedione.

During testosterone (**2**) transformation the ratio of androstenedione (**3**) to testosterone increased, as the time of reaction is prolonged (Table 1), and besides **3** was hydroxylated faster than **2**, so the products obtained after transformation of **2** were the results of hydroxylation of both **2** and **3**. Higher contents of 6β-alcohols **8** and **9** in products obtained from **2** than from **3** and the lack of 15α-hydroxytestosterone in the mixture of products after 12 h transformation of testosterone suggest that testosterone was hydroxylated only at 6β position.

In the transformation of 17α-hydroxyprogesterone a mixture of many products, some of them less polar than the substrate, were formed. Only two, more polar products: 6β,17α-dihydroxyprogesterone and 15α,17α-dihydroxyprogesterone were identified.

The results presented above show a relationship between the structure of the substrate and site of the introduced hydroxyl groups. The formation of a 12β,15α-diol **7**, as a major product of 4-androsten-3-one transformation indicates that in the presence of 4-ene-3-oxo system in the substrates, hydroxylation of 12β and 15α positions occurs. All the 4-ene-3-oxo substrates, except for testosterone, were hydroxylated at 15α position, but only the substrates with oxygen function at C-17 were hydroxylated at 6β site. It is quite likely that the relationship between the presence of oxygen function at C-17 in the substrate and hydroxylation at 6β position was associated with the induction of 6β-hydroxylase activity by these substrates.

As has been found, the transformation is carried out faster when its time is prolonged (Table 1), e.g. after 12 h of androstenedione incubation, 22% of the substrate was hydroxylated, whereas after 24 h almost complete transformation of this substrate occurred, which proves that the presence of the substrate induces hydroxylase activity.

It was also observed [18] that androstenolone was entirely hydroxylated in *F. culmorum* culture at 7α-allylic, axial position. The vicinity of 6β and 7α sites and their axial, allylic feature, suggest that they can be hydroxylated by the same enzyme. One might expect, that the products of androstenedione transformations by *F. culmorum* should have been higher in 6β alcohols **8** and **9** when androstenolone was used as an inductor, than without it, if 6β and 15α androstenedione hydroxylations had been carried out by different enzymes and 7α- and 6β-allylic hydroxylation by the same enzyme.

After 12 h of androstenedione incubation, 55% of hydroxylation products were formed in the *F. culmorum* culture induced by androstenolone, whereas only 22% of the products were produced by non-induced microorganisms (Table 1). This indicates that androstenolone induces the androstenedione hydroxylase activity. Similar percentages of 6β- and 15α-alcohols formed in induced and non-induced cultures suggest that the 6β and 15α positions may have been hydroxylated by the same enzyme. Additionally, androstenolone transformations by *F. culmorum*, with androstenedione used as an inductor, had the same selectivity; the only product was 7α-alcohol, and the inductor increased the percentage of the transformed substrate as compared to that obtained in the non-induced culture [18].

The influence of the function groups of a substrate on the position of hydroxylation in *F. culmorum* culture was likely due to contribution of these groups to the formation of the enzyme–substrate complex. The directing influence of substituents in the substrate molecule resulting from their contribution to the formation of the enzyme–substrate complex was first proposed to explain 6β- and 11α-hydroxylation of steroids by *Aspergillus tamarii* [19]. Analyzing Dreiding's models we can see that 12β- and 15α-hydrogen can occupy a similar position against the hydroxylase active center in the two complexes formed by 3-oxo group, varying in substrate orientation by rotation (180°) along C_3–C_{17} axis. If 3-oxo-group in the enzyme–substrate complex is replaced by oxygen function at C-17 and orientation of the substrate molecule is the same as in the complex for 12β-hydroxylation, then 6β hydrogen can have the same position against the active center as 15α or 12β hydrogen in previous complexes.

Hydroxylation of 6β axial positions in the 4-ene-3-ketones is favored by the activating vicinity of electrons π. Selective 6β hydroxylation of testosterone is likely due to the directing influence of 17β-hydroxyl group and allylic position of 6β axial hydrogen. Lower amounts of 6β alcohols obtained from androstenedione than testosterone (although the observed [20] directing effect of the carbonyl group is similar or greater than that of the hydroxyl group) is likely due to activating of C-15 positions by the presence of the 17-oxo group (in its enolic form). 15α-Hydroxylation of the substrates without a carbonyl group at C-17 was also observed, e.g. 15α-hydroxyprogesterone was a major product of progesterone transformation, but transformation of this substrate took twice as long as that of androstendione.

The course of transformations of the investigated substrates by *F. culmorum* indicates the influence of a substrate structure on the site of hydroxylation. The results obtained in the present study confirm that directing effect of substituents observed in transformation e.g. by *Calonectria decora* [21, 22] and *Gnomonia fructicola* [23] is connected with contribution of the group present in the substrate to the formation of the enzyme–substrate complex.

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