Transformations of Steroid Esters by *Fusarium culmorum*

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Z. Naturforsch. **61c**, 809–814 (2006); received April 6/May 15, 2006

The course of transformations of the pharmacological steroids: testosterone propionate, 4-chlorotestosterone acetate, 17β-estradiol diacetate and their parent alcohols in *Fusarium culmorum* AM282 culture was compared. The results show that this microorganism is capable of regioselective hydrolysis of ester bonds. Only 4-ene-3-oxo steroid esters were hydrolyzed at C-17. 17β-Estradiol diacetate underwent regioselective hydrolysis at C-3 and as a result, estrone – the main metabolite of estradiol – was absent in the reaction mixture.

The alcohols resulting from the hydrolysis underwent oxidation at C-17 and hydroxylation. The same products (6β- and 15α-hydroxy derivatives) as from testosterone were formed by transformation of testosterone propionate, but the quantitative composition of the mixtures obtained after transformations of both substrates showed differences. The 15α-hydroxy derivatives were obtained from the ester in considerably higher yield than from the parent alcohol.

The presence of the chlorine atom at C-4 markedly reduced 17β-saponification in 4-chlorotestosterone acetate. Only 3β,15α-dihydroxy-4α-chloro-5α-androstan-17-one (the main product of transformation of 4-chlorotestosterone) was identified in the reaction mixture. 6β-Hydroxy-4-chloroandrostenedione, which was formed from 4-chlorotestosterone, was not detected in the extract obtained after conversion of its ester.

Key words: Fusarium culmorum, Biotransformation, Steroids

Introduction

The hydroxylation of steroids is important due to its physiological role in mammalian organisms. Recognition of transformations of steroid hormones and their derivatives, especially those used as drugs, in the culture of some microorganisms can provide useful information about metabolic processes of xenobiotics in mammals, in connection with the well-accepted concept of "microbial models of mammalian metabolism" (Griffiths *et al.*, 1991; Azerad, 1999).

In our previous work we investigated transformations of the 4-en-3-one steroid hormones and their derivatives by means of *Fusarium culmorum* AM282 (Kołek and Świzdor, 1998; Świzdor and Kołek, 2005). We showed that the reactions were of significant regio- and stereoselectivity and that the position of the introduced hydroxy group depended on the substrate structure. The transformations of testosterone and androstenedione (compounds differing only in the kind of an oxygen function at C-17) yielded the same products: 6β-hydroxyandrostenedione, 6β-hydroxytestosterone, 15α-hydroxyandrostenedione and 15α-hydroxytestosterone, but the obtained quantities of 6β- and 15α-alcohols varied, depending on the substrate used. During transformations, apart from hydroxylation, ketone-alcohol interconversion at C-17 occurred for the substrates as well as their hydroxylation products (Kołek and Świzdor, 1998). However, the 17-keto group wasn't converted back to the hydroxy group during transformation of 4-chlorotestosterone. This compound was predominantly hydroxylated at 15α-position, but the reaction was accompanied by the reduction of the 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 (Świzdor and Kołek, 2005).

In this work, in order to examine the influence of the ester group on the course of transformation, we carried out the bioconversion of the pharmacological steroids: testosterone propionate (**1**), 4-chlorotestosterone acetate (**8**), and 17β-estradiol diacetate (**12**) in *F. culmorum* culture.

The esters of steroidal alcohols are used as drugs with prolonged action in comparison with free alcohols (Zeelen, 1990). Short-chain esters (*e.g.* acetate, propionate) give rise to short-acting steroids, whereas long-chain esters (*e.g.* decanoate, enanthate) are long-acting compounds. Esterification

0939–5075/2006/1100–0809 $ 06.00

of the 17-hydroxy group delays biodegradation of orally administrated testosterone to biologically inactive keto steroids (Shahidi, 2001). Testosterone propionate (**1**), like testosterone itself, has androgenic and anabolic activity. The analogues of testosterone substituted in the 4-position (especially 4-halogenated compounds) are highly anabolic. 4-Chlorotestosterone [frequently available as 4-chlorotestosterone acetate (**8**)] is known on the black market as Clostebol, Macrobin, Steranobol, and Turinabol and can be illegally used, *e.g.* in cattle as growth promoting agent or as aggression and mass promoter in racing animals. 17β-Estradiol 3-acetate provides improved bioavailability of estrogen when orally administered to a human female in needs of estrogen replacement therapy in conditions of inadequate estrogen production.

The metabolism of testosterone and 4-chlorotestosterone has been investigated in various tissues *in vivo* and *in vitro* in several animals and in clinical studies in humans (Rendic *et al*., 1999; Tamura *et al*., 1996; Williams *et al*., 2000; Costegnaro and Sala, 1973; Cartoni *et al*., 1983; Schänzer and Donike, 1993; André *et al*., 1994; Hendriks *et al*., 1994; Leyssens *et al*., 1994; Le Bizec *et al*., 1998; Walshe *et al*., 1998; Leung *et al*., 2005). Depending on the method of administration, different metabolites had been found over a period of time. The typical reactions of phase I of metabolism of these steroids involve oxidation at C-17, reduction at C-3 and C-17, reduction of the double 4,5 bond, hydroxylation (mainly in B or D ring) and epimerisation. The major pathway of testosterone and 4-chlorotestosterone oxidation is 6β-hydroxylation. Several studies suggested the formation of other hydroxy derivatives, however the position of the hydroxylation could not be unambiguously assigned due to the lack of the specific reference standards.

Materials and Methods

Microorganism

The microorganism *Fusarium culmorum* AM282 used in this study was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław, Poland. It was isolated from *Zea mays*.

Conditions of cultivation and transformation

The strain of *F. culmorum* was maintained on Sabouraud 4% dextrose agar slope and freshly subcultured before use in the transformation experiments.

300 ml Erlenmeyer flasks, each containing 100 ml of sterile medium consisting of 3% glucose and 1% peptone, were inoculated with a suspension of *F. culmorum* and then incubated for 3 d at 20 °C on a rotary shaker. After this growth period of the microorganism, 20–25 mg of a substrate in 0.5 ml of acetone were added to each of the cultures, and the transformation was continued under the same conditions, as long as the contents of substrate in the reaction mixture underwent a change. Conversion of the substrates was monitored by TLC and GC.

Product isolation and analysis

The samples (5 ml) of the conversion medium were taken every 12 h, extracted with chloroform, and analyzed. The identification of the metabolites of testosterone and 4-chlorotestosteron esters **1** and **8** was performed by comparison of their R_f and R_t values with those of previously prepared specific reference standards (Kołek and Świzdor, 1998; Świzdor and Kołek, 2005). TLC analysis was carried out using Merck Kieselgel 60 F_{254} plates with hexane/acetone (1:1 or 2:1 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^{-1}), equipped with a HP-5 column (cross-linked 5% Ph-Me-Siloxane, 25 m × 0.32 mm × 0.52 μm film thickness) for **1** (temperature conditions: 220 °C – 1 min, 6 °C min^{-1} to 250 °C, 2 °C min^{-1} to 300 °C – 5 min), for **10** (220 °C – 1 min, 8 °C min^{-1} to 280 °C, 10 °C min^{-1} to 300 °C – 5 min), and for **12** (220 °C – 1 min, 10 °C min^{-1} to 270 °C, 4 °C min^{-1} to 300 °C – 5 min), or a Thermo TR-1MS column (60 m × 0.32 mm × 0.25 μm film thickness) for **8** (220 °C – 1 min, 5 °C min^{-1} to 300 °C – 5 min). Column chromatography (for **10** and **12**) was performed using silica gel and a hexane/acetone mixture (1:1 v/v) as eluent. Structures of metabolites **11** and **13** were determined by means of ^{1}H NMR (the spectra were recorded on a DRX 300 MHz Bruker spectrometer and measured in $CDCl_3$ with TMS as an internal standard).

Table I. Biotransformation of steroids by *Fusarium culmorum*.

Substrate	Product	%[a]	R_t [min]
Testosterone propionate (**1**)	Testosterone (**2**)	8	17.24
	Androstenedione (**3**)	22	16.94
	6β-Hydroxyandrostenedione (**5**)	12	20.63
	6β-Hydroxytestosterone (**4**)	6	20.96
	15α-Hydroxyandrostenedione (**7**)	19	22.71
	15α-Hydroxytestosterone (**6**)	33	23.32
4-Chlorotestosterone acetate (**8**)	Recovered substrate (**8**)	75	12.93
	3β,15α-Dihydroxy-4α-chloro-5α-androstan-17-one (**9**)	23	9.98
17β-Estradiol (**10**)	Recovered substrate (**10**)	77	10.43
	Estrone (**11**)	22	10.31
17β-Estradiol diacetate (**12**)	Recovered substrate (**12**)	8	13.72
	17β-Estradiol 17-acetate (**13**)	92	12.53

[a] Yield of the chloroform extract determined by GC.

^{1}H NMR data of metabolite **11**: δ (ppm) = 0.90 (s, 18-H_3), 6.57 (s, 4-H), 6.62 (dd, J = 2.3, 8.3 Hz, 2-H), 7.14 (d, J = 8.3 Hz, 1-H).

^{1}H NMR data of metabolite **13**: δ (ppm) = 0.83 (s, 18-H_3) 2.06 (s, 17β-OAc), 4.69 (t, J = 8.6 Hz, 17α-H), 6.56 (s, 4-H), 6.62 (d, J = 8.3 Hz, 2-H), 7.14 (d, J = 8.3 Hz,1-H).

The yields (determined by GC analysis of chloroform extract) and R_t values of metabolites are shown in Table I.

Results and Discussion

In order to examine the influence of an ester group on the course of transformation in *Fusarium culmorum* AM282 culture, the transformations of testosterone propionate (**1**), 4-chlorotestosterone acetate (**8**), 17β-estradiol diacetate (**12**) and their parent alcohols were compared.

Testosterone propionate (**1**) was extensively metabolized, unlike what can be observed for two other esters (Fig. 1). The parent drug was not detected in the reaction mixture after 36 h of incubation. Time course experiments evidently indicated (data not presented) that the first stage of the transformation was a hydrolysis of the ester bond (the control experiment with the suspension of autoclaved mycelium of *F. culmorum* did not give a transformation product and confirmed that **1** is stable under the incubation condition). Testosterone (**2**), which was a result of this reaction, underwent oxidation at C-17 and 6β- or 15α-hydroxylation. Therefore, the biotransformations of testosterone and its ester **1** gave mixtures of the same metabolites: 6β-hydroxytestosterone (**4**), 6β-hydroxyandrostenedione (**5**), 15α-hydroxytestosterone (**6**) and 15α-hydroxyandrostenedione (**7**). However, the comparative analysis of percentage composition of mixtures obtained after transformations of both substrates indicated quantitative differences. Fig. 2 shows the contents of 6β- and 15α-hydroxy metabolites in reaction mixtures obtained from transformations of testosterone and its ester **1** in which the ratios of testosterone to androstenedione were comparable. Considerably smaller amounts of 6β-alcohols were formed from testosterone propionate (**1**) than testosterone (18% and 40%, respectively). It is worth noting that the main hydroxylation product of **1** was 15α-hydroxytestosterone (**6**) (33%). Because it was reported that reduction of the carbonyl group at C-17 to the β-alcohol occurred in the products of androstenedione hydroxylation (Kołek and Świzdor, 1998), it is possible that formation of androstenedione (**3**) occurs more efficiently from testosterone ester **1** than from unmodified testosterone. This result was unexpected, since the 17-*O*-modification is known to protect from rapid 17-metabolism. So far, the higher formation of androstenedione from testosterone esters than testosterone has been observed in cultured human dermal fibroblast (Tamura *et al*., 1996).

4-Chlorotestosterone acetate (**8**) showed very low susceptibility to enzymatic hydrolysis. After 14 days of transformation, 75% of the unchanged substrate was recovered. Only 3β,15α-dihydroxy-4α-chloro-5α-androstan-17-one (**9**) [the main

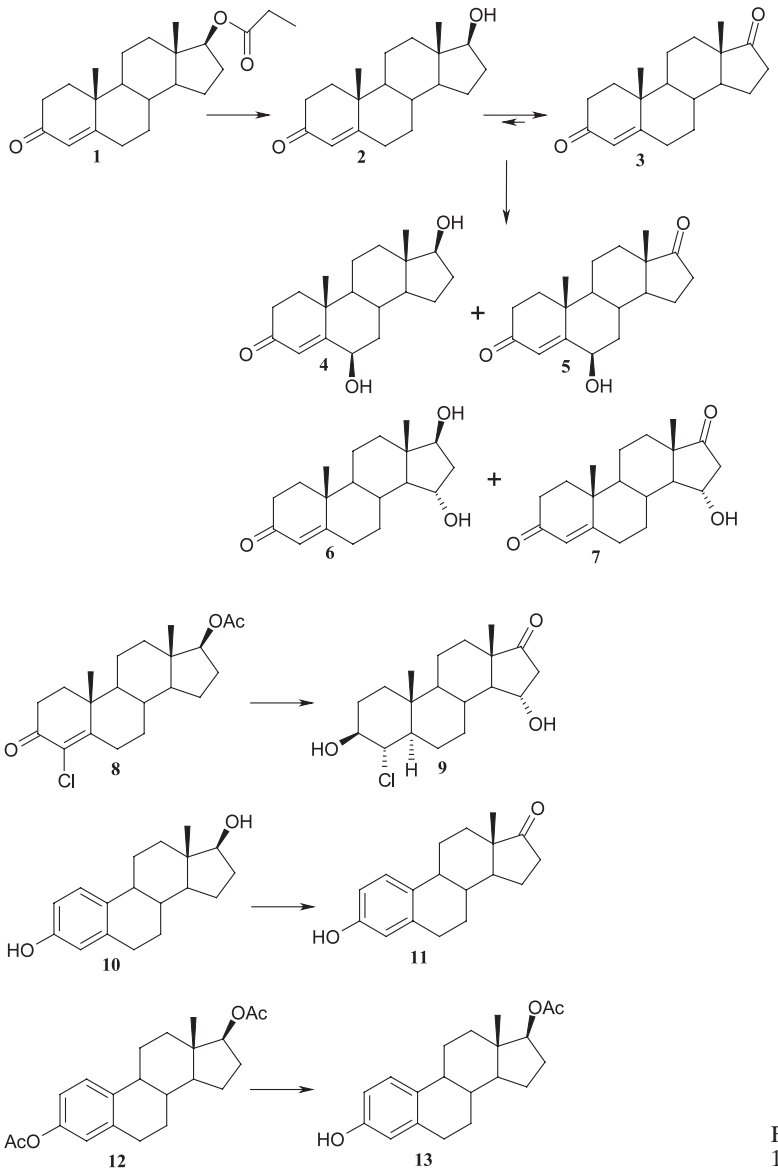

Fig. 1. Metabolism of steroid esters and 17β-estradiol by *Fusarium culmorum.*

product of transformation of 4-chlorotestosterone (Świzdor and Kołek, 2005)] was identified in the reaction mixture. 6β-Hydroxy-4-chloroandrostenedione, which was formed from 4-chlorotestosterone (Świzdor and Kołek, 2005), was not detected in the extract obtained after conversion of the ester **8**.

The results obtained indicated that 17β-estradiol (**10**) was inert to hydroxylation by *F. culmorum*. After 14 days of transformation, the only product (22%) was estrone (**11**). This metabolite is the result of oxidation of the hydroxy group at C-17 of estradiol. The structure of this product was deduced from its ^{1}H NMR spectrum, which showed no signal of the 17α-proton at 3.73 ppm, and the down-field shift of the 18-H_3 signal (about 0.12 ppm with respect to the substrate). The oxidation to estrone is a main metabolism pathway of estradiol in the liver (Zeelen, 1990) and in the human skin (Liu *et al.*, 1994).

17β-Estradiol (**10**) was not released from 17β-estradiol diacetate (**12**). This substrate underwent

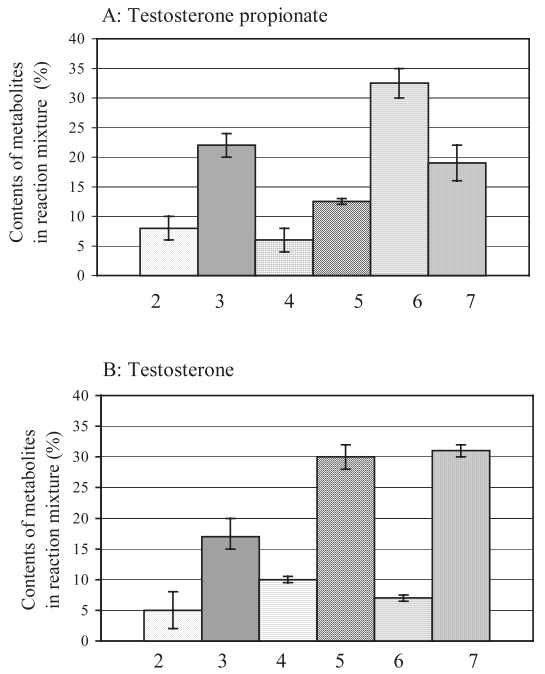

Fig. 2. Comparison of reaction mixture composition obtained from transformation of testosterone propionate and testosterone. 2, Testosterone; 3, androstenedione; 4, 6β-hydroxytestosterone; 5, 6β-hydroxyandrostenedione; 6, 15α-hydroxytestosterone; 7, 15α-hydroxyandrostenedione. The vertical bars represent the deviation from the mean calculated from three independent experiments.

regioselective hydrolysis at C-3. The 17β-ester resisted cleavage. The structure of the sole product 17β-estradiol 17-acetate (**13**) (92%) was deduced from its ^{1}H NMR spectrum, which showed no signal of 3β-OAc at 2.28 ppm, and the up-field shift of phenolic ring protons. The examination of the literature reveals that hydrolysis of **12** proceeded with regioselectivity such as that observed for lipase from *Candida cylindracea* (yeast lipase) (Njar and Capsi, 1987). The regioselectivity of saponification by *F. culmorum* creates a possibility for the application of this biocatalyst in protecting group chemistry.

To sum up, although the first stage of the microbial transformation of steroid esters was a hydrolysis of the ester bond and the released alcohol underwent further conversion, we observed the difference in the metabolism of steroid alcohols and their esters. These data proved that the prediction of a metabolic pathway could fail even in the case of apparently simplest example, and it needs to be validated using living organisms.

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