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Application of α- and β-naphthoflavones as monooxygenase inhibitors of *Absidia coerulea* KCh 93, *Syncephalastrum racemosum* KCh 105 and *Chaetomium* sp. KCh 6651 in transformation of 17α-methyltestosterone

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ABSTRACT

In this work, 17α-methyltestosterone was effectively hydroxylated by *Absidia coerulea* KCh 93, *Syncephalastrum racemosum* KCh 105 and *Chaetomium* sp. KCh 6651. *A. coerulea* KCh 93 afforded 6β-, 12β-, 7α-, 11α-, 15α-hydroxy derivatives with 44%, 29%, 6%, 5% and 9% yields, respectively. *S. racemosum* KCh 105 afforded 7α-, 15α- and 11α-hydroxy derivatives with yields of 45%, 19% and 17%, respectively. *Chaetomium* sp. KCh 6651 afforded 15α-, 11α-, 7α-, 6β-, 9α-, 14α-hydroxy and 6β,14α-dihydroxy derivatives with yields of 31%, 20%, 16%, 7%, 5%, 7% and 4%, respectively. 14α-Hydroxy and 6β,14α-dihydroxy derivatives were determined as new compounds. Effect of various sources of nitrogen and carbon in the media on biotransformations were tested, however did not affect the degree of substrate conversion or the composition of the products formed. The addition of α- or β-naphthoflavones inhibited 17α-methyltestosterone hydroxylation but did not change the percentage composition of the resulting products.

1. Introduction

Steroid compounds possess a wide range of biological properties essential for the pharmaceutical industry. They exhibit anti-inflammatory, immunosuppressive, diuretic, anabolic and contraceptive properties. They are used in the treatment of breast cancer, prostate cancer, osteoporosis, adrenal insufficiency, in the prevention of heart disease, as anti-obesity, antifungal and antidepressant agents, and play a key role in the management of human fertility [1–5]. Steroids are a group of compounds that have basic functions in the life processes of eukaryotic organisms and are mainly used in the pharmaceutical industry. Due to hydroxylation of steroids using microbiological monooxygenases (cytochrome P-450 enzymes), compounds with the desired biological activity can be obtained [2,3,6–8]. Some of the advantages of biotransformation are selectivity of enzymes, mild process conditions and biodegradability of reagents [9]. The biohydroxylation process is characterised by high regio- and stereospecificity, which makes it possible to obtain compounds difficult to obtain by classical synthesis [10,11].

In this work, 17α-methyltestosterone (**1**) was used as a substrate. This compound is characterised by high androgenic activity and is used in the treatment of endocrine diseases. The widespread use of this compound results from its better absorption from the gut in relation to testosterone. Due to this property, it is also used illegally by athletes [12,13]. Verification, whether the tested athlete used an illicit agent, is performed by gas chromatography (GC) or high performance liquid chromatography (HPLC) analysis by comparing the compounds present in his or her blood with the standards present in the base of prohibited compounds [1,14]. Therefore it is extremely important to identify and produce new derivatives that will expand the base of known anabolics.

17α-Methyltestosterone (**1**) may undergo similar transformations in the cultures of microorganisms as in the human body (products of both may be identical). 17α-Methyltestosterone is rapidly metabolised in the human body, and therefore monitoring the abuse of this steroid usually involves detecting its products [12]. Two main products of this compound's metabolism have been identified in humans: 17α-methyl-5α- and 17α-methyl-5β-androstan-3α,17β-diol [13]. In other mammals, hydroxyderivatives were also identified, suggesting that these derivatives may be formed in humans. During *in vivo* studies on horses, cows and rabbits, in addition to the ring A reduction products, the effects of monooxygenases (6-, 15- and 16-hydroxy-17-methyltestosterone) were also observed [15–17].

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17α-Methyltestosterone is used in fish (*Danio rerio* and *Oncorhynchus mykiss*) farming to induce male monosex cultures [18,19]. Male individuals are economically advantageous because they have higher growth rates not inhibited by energy losses associated with the development of female gonads and eggs [20]. However, intensive use of this steroid for tilapia (*Oreochromis niloticus*) still raises concerns about risks to the health of consumers and the natural environment [20]. Although 17α-methyltestosterone (**1**) effects on industrial species are investigated, possible negative effects on non-target species are not fully understood. Several authors have described the negative effects of this compound exposure on sexual development and reproduction of juvenile and adult individuals of various fish species showing fertility decline, development of male secondary sex characteristics and inhibition of gonadal development in females [21–23].

2. Materials and methods

2.1. Materials

The substrate, 17α-methyl-17β-hydroxyandrost-4-en-3-one (**1**), was purchased from Sigma-Aldrich. α- and β-Naphthoflavone and their derivatives (**11**–**18**) were obtained from relevant 2′-hydroxychalcones according to the previously described method [24,25]. Two grams of the substrate was dissolved in DMSO (75 ml) with the addition of I_2 (0.1 eqv.), and the reaction mixture was refluxed for 4 h. Crystallization from ethanol afforded pure flavones (**11**–**18**). 2′-Hydroxynaphthochalcones were obtained from 2′-hydroxyacetophenone and aldehyde derivatives with the methods described previously [24,26,27]. Spectral data of all obtained chalcones and flavones are indentical to those previously published [24,28–35]. The strains of *Absidia coerulea* KCh 93, *Syncephalastrum racemosum* KCh 105, and *Chaetomium* sp. KCh 6651 used in this study were obtained from the collection of the Department of Chemistry, Wrocław University of Environmental and Life Sciences, Poland. They were isolated from a forest environment (in southern Poland) from dead parts of leafy plants. The strains were maintained on Sabouraud 4% dextrose-agar slopes and freshly subcultured before use in the transformation experiments.

2.2. Screening procedure

Erlenmeyer flasks (300 ml), each containing 100 ml of the medium consisting of 3 g of glucose and 1 g of aminobac dissolved in water, were inoculated with a suspension of microorganisms and then incubated for 3 days at 25 °C on a rotary shaker. After full growth of the culture (about 12 g of cell dry weight/l) 20 mg of a substrate dissolved in 1 ml of acetone was added. After the first, third, sixth and ninth days of incubation under the above conditions, portions of 10 ml of the transformation mixture were taken out and extracted with $CHCl_3$ (3 × 10 ml). The extracts were dried over $MgSO_4$, concentrated *in vacuo* and analysed by GC. All the experiments were repeated three times.

2.3. Preparative biotransformation

The same transformations were performed on the preparative scale in 2000 ml flasks, each containing 500 ml of the cultivation medium. The cultures were incubated under the same conditions, and then 200 mg of substrate dissolved in 2 ml of acetone was added to the grown cultures. After 3 days of incubation, the mixtures were extracted with $CHCl_3$ (3 × 300 ml), dried over $MgSO_4$ and concentrated *in vacuo*. The transformation products were separated by column chromatography and analysed by TLC, GC and GC–MS.

2.4. Effects of nitrogen sources

Erlenmeyer flasks (300 ml), each containing 100 ml of the medium consisting of 3 g of glucose and 1 g of tested nitrogen source (aminobac, peptone or yeast extract dissolved in water), were inoculated with a suspension of microorganisms. After a 3-day incubation at 25 °C on a rotary shaker, the experiments were carried out according to the procedure described in Section 2.2.

2.5. Effects of carbon sources

Erlenmeyer flasks (300 ml), each containing 100 ml of the medium consisting of 3 g of tested monosaccharide (D-ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, L-glucose, D-mannose and D-fructose) or disaccharide (lactose, maltose, sucrose) and 1 g of aminobac dissolved in water, were inoculated with a suspension of microorganisms and then incubated for 3 days at 25 °C on a rotary shaker. The experiments were carried out according to the procedure described in Section 2.2.

2.6. Biotransformation with inhibitors

Biotransformations were carried out under the same conditions as described in Section 2.2. After full growth of the selected culture 2 mg of inhibitor (one of α- or β-naphthoflavones) dissolved in 0.5 ml of acetone was added. After one hour, 20 mg of a substrate dissolved in 1 ml of acetone was added. The reactions were analysed as in Section 2.2.

2.7. Analytical methods

The course of biotransformation was analysed using TLC. The composition of product mixtures was established by GC. Products were separated by column chromatography using silica gel (Kieselgel 60, 230–400 mesh, Merck) and a hexane/acetone mixture (2:1, v/v) as eluent. Analytical TLC was carried out on silica gel G (Merck). Compounds were detected by spraying the plates with a H_2SO_4/CH_3OH mixture (1:1, v/v). GC analysis was performed using a Hewlett-Packard 5890A (Series II) GC instrument fitted with a flame ionisation detector (FID). A DB-5MS (crosslinked phenyl methyl siloxane) capillary column (30 m × 0.32 mm × 0.25 μm) was used to determine the composition of product mixtures. The following temperature programme was used: 200 °C (0 min)/10 °C/min/270 °C (0 min)/30 °C/min/300 °C (5 min). The NMR spectra were recorded on a DRX 500 MHz Bruker spectrometer and measured in $CDCl_3$ or THF-d8. MS analyses were performed on a Varian Chrompack GC CP-3800 Saturn 2000GC/MS/MS with an ionizing energy of 70 eV. Elemental analysis was carried out on the Vario EL III CHNS (Elementor).

2.8. Identification of isolated products

The products' structures were determined by means of elemental analysis, ^{1}H NMR, ^{13}C NMR and correlation spectroscopy. ^{13}C NMR spectra of all the products obtained are summarised in Table 1.

2.8.1. 7α-Hydroxy-17α-methyltestosterone (**2**)

White crystalline solid, ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.90 (s, 3H, 18-C*H*$_3$); 1.20 (s, 3H, 19-C*H*$_3$); 1.23 (s, 3H, 17α-C*H*$_3$); 3.96 (q, 1H, *J* = 2.7 Hz, *H*-7β) 5.79 (s, 1H, *H*-4).

Table 1
^{13}C NMR chemical shifts of products in $CDCl_3$.

Atom number	Products									
	2	**3**	**4**	**7**	**5**	**5***	**6**	**8**	**9**	**10**
1	38.7	38.9	35.8	34.1	38.2	38.5	32.8	33.5	28.4	33.6
2	35.5	34.2	32.8	32.9	34.3	35.1	35.7	34.0	34.0	34.2
3	199.0	200.4	199.6	199.7	200.3	198.9	199.5	199.6	199.1	200.3
4	126.9	124.5	123.8	124.1	126.4	126.4	124.1	123.9	126.9	126.3
5	167.8	171.3	171.0	171.2	168.2	169.4	170.6	170.7	168.6	167.8
6	41.1	33.7	34.0	32.8	73.1	73.4	22.9	32.6	31.7	73.4
7	67.8	31.3	32.3	32.2	38.9	40.1	38.2	38.8	25.0	39.0
8	40.7	36.2	36.1	35.9	30.6	31.8	35.5	35.8	38.3	33.4
9	45.4	59.1	53.8	54.3	53.6	55.3	52.4	46.6	76.6	53.8
10	38.6	40.1	38.7	38.9	38.1	39.1	38.5	38.9	44.5	37.2
11	20.5	69.3	20.5	20.7	20.6	21.8	29.5	19.9	26.1	19.8
12	31.1	43.8	31.5	31.5	31.4	32.8	73.3	30.6	27.0	33.3
13	45.3	46.2	46.6	44.9	45.4	46.6	49.4	49.8	45.2	49.9
14	44.6	49.5	57.9	54.8	50.1	51.6	48.7	84.1	43.8	84.3
15	22.7	23.2	72.3	69.2	23.1	24.4	23.0	25.6	22.9	23.7
16	34.0	37.5	50.6	52.0	37.1	39.9	31.1	39.5	38.8	38.3
17	81.6	81.1	79.1	81.4	81.5	80.9	81.8	81.8	81.5	81.8
18	13.8	15.1	15.4	16.7	14.0	14.7	8.5	18.8	13.0	18.9
19	19.1	18.4	17.5	17.5	19.6	19.9	17.3	17.2	19.8	19.4
20	26.0	25.9	26.2	25.4	25.7	26.6	25.7	29.3	26.1	25.7

5* in THF-d8.

*2.8.2. 11α-Hydroxy-17α-methyltestosterone (**3**)*

White solid, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.92 (s, 3H, 18-C*H*$_3$); 1.22 (s, 3H, 17α-C*H*$_3$); 1.32 (s, 3H, 19-C*H*$_3$); 4.05 (td, 1H, *J* = 10.3, 4.7 Hz, *H*-15β); 5.72 (s, 1H, *H*-4).

*2.8.3. 15α-Hydroxy-17α-methyltestosterone (**4**)*

White crystalline solid, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.91 (s, 3H, 18-C*H*$_3$); 1.21 (s, 3H, 19-C*H*$_3$); 1.35 (s, 3H, 17α-C*H*$_3$); 4.09 (td, 1H, *J* = 9.3, 3.4 Hz, *H*-15β); 5.73 (s, 1H, *H*-4).

*2.8.4. 6β-Hydroxy-17α-methyltestosterone (**5**)*

White crystalline solid, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.94 (s, 3H, 18-C*H*$_3$); 1.40 (s, 3H, 19-C*H*$_3$); 1.22 (s, 3H, 17α-C*H*$_3$); 4.35 (t, 1H, *J* = 2.8 Hz, *H*-6α); 5.82 (s, 1H, *H*-4). ^1H NMR (500 MHz, THF-D8) δ (ppm): 0.90 (s, 3H, 18-C*H*$_3$); 1.13 (s, 3H, 17α-C*H*$_3$); 1.39 (s, 3H, 19-C*H*$_3$); 3.28 (s, 1H, 17β-O*H*); 4.17 (q, 1H, *J* = 2.8 Hz, *H*-6α); 4.29 (dd, 1H, *J* = 2.7, 1.4 Hz, 6β-O*H*); 5.66 (s, 1H, *H*-4).

*2.8.5. 12β-Hydroxy-17α-methyltestosterone (**6**)*

Bright yellow thick oil, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.95 (s, 3H, 18-C*H*$_3$); 1.19 (s, 3H, 19-C*H*$_3$); 1.33 (s, 3H, 17α-C*H*$_3$); 3.73 (dd, 1H, *J* = 16.8, 7.2 Hz, *H*-12α); 5.71 (s, 1H, *H*-4).

*2.8.6. 15β-Hydroxy-17α-methyltestosterone (**7**)*

White solid, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.15 (s, 3H, 17α-C*H*$_3$); 1.18 (s, 3H, 18-C*H*$_3$); 1.23 (s, 3H, 19-C*H*$_3$); 4.20 (ddd, 1H, *J* = 7.9, 5.5, 2.4 Hz, *H*-15α); 5.73 (s, 1H, *H*-4).

*2.8.7. 14α-Hydroxy-17α-methyltestosterone (**8**)*

Bright yellow thick oil, Anal. Calcd. for $C_{20}H_{30}O_3$: C 75.43, H 9.50%; found: C 75.39, H 9.52%. Mass spectrum indicated a molecular ion at *m*/*z* 319.34 $[M]^+$; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.03 (s, 3H, 18-C*H*$_3$); 1.21 (s, 3H, 19-C*H*$_3$); 1.49 (s, 3H, 17α-C*H*$_3$); 5.72 (s, 1H, *H*-4).

*2.8.8. 9α-Hydroxy-17α-methyltestosterone (**9**)*

Bright yellow thick oil, ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 0.91 (s, 3H, 18-C*H*$_3$); 1.33 (s, 3H, 19-C*H*$_3$); 1.24 (s, 3H, 17α-C*H*$_3$); 5.86 (d, 1H, *J* = 0.9 Hz, *H*-4).

*2.8.9. 6β,14α-Dihydroxy-17α-methyltestosterone (**10**)*

Bright yellow thick oil; Anal. Calcd. for $C_{20}H_{30}O_4$: C 71.82, H 9.04%; found: C 71.76, H 9.07%. Mass spectrum indicated a molecular ion at *m*/*z* 335.33 $[M]^+$; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.07 (s, 3H, 18-C*H*$_3$); 1.25 (s, 3H, 19-C*H*$_3$); 1.51 (s, 3H, 17α-C*H*$_3$); 4.42 (t, 1H, *J* = 2.8 Hz, *H*-6α); 5.81 (s, 1H, *H*-4).

3. Results and discussion

Biotransformations of 17α-methyltestosterone (**1**) were performed by *Syncephalastrum racemosum* KCh 105, *Absidia coerulea* KCh 93, *Chaetomium* sp. KCh 6651, which were chosen on the basis of their known ability to transform steroid compounds effectively. The substrate (**1**) was hydroxylated in high yield by all selected strains, however none of the cultures characterised high selectivity of a single hydroxylation process. Mixtures of many products were obtained in all described biotransformations (Table 2). Structures of all obtained compounds were determined by spectroscopic methods (NMR, GC–MS) For all obtained products a characteristic shift of a single (two for compound **10**) carbon signal downfield (67–85 ppm) on a ^{13}C NMR spectra was observed, indicating introduction of a hydroxyl group to a structure. Similar characteristic changes were observed on ^1H NMR spectra of products, where introduction of a hydroxyl group resulted in new signals downfield (3.7–4.5 ppm). In case of products **8** and **9**, no characteristic changes in chemical shifts of signals on ^1H NMR spectra were observed, albeit signals at 84.1 and 76.6 on ^{13}C NMR spectra, respectively, indicating hydroxylation of one of the tertiary carbons in the substrate (Table 1). Further analysis of correlation spectra (COSY, HMQC, HMBC) and comparison with NMR spectra of 17α-methyltestosterone (**1**) allowed unambiguous structure elucidation of obtained products including hydroxyl group orientation. Spectral data of new compounds were additionally compared with similar molecules [36], for known compounds spectral data obtained were in agreement with literature values [9,37–41].

The highest selectivity of hydroxylation was observed in the culture of the *S. racemosum* KCh 105. Three products were obtained: 7α-hydroxy-17α-methyltestosterone (**2**), **4, 3** in yields 45%, 19%, 17%, respectively (Fig. 1). In the literature, strains from this species are described as effective biocatalysts due to their highly active monooxygenases. The enzymes allow them to trans-

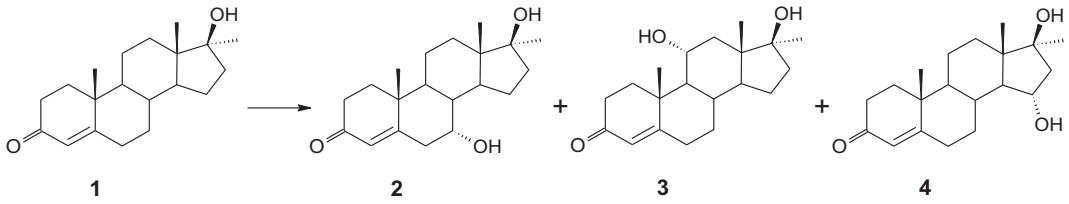


Fig. 1. Transformation of 17α-methyltestosterone (**1**) by *Syncephalastrum racemosum* KCh 105.

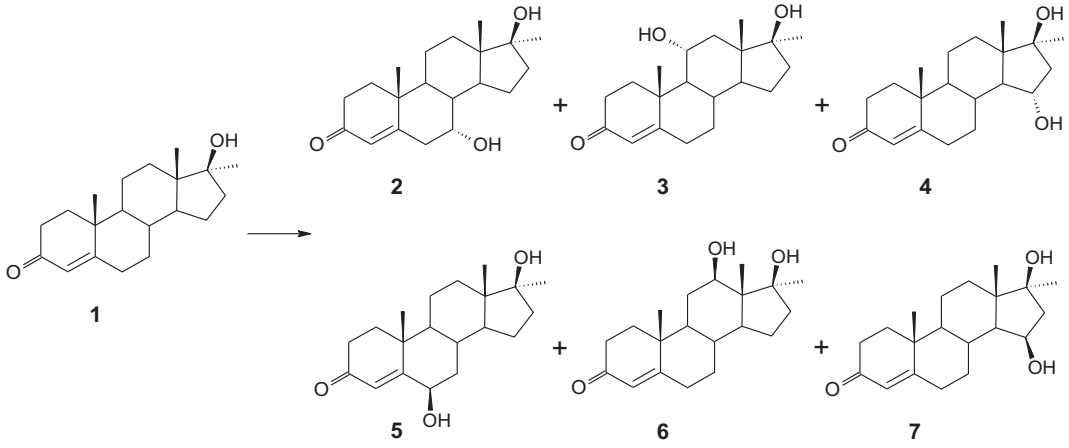


Fig. 2. Transformation of 17α-methyltestosterone (**1**) by *Absidia coerulea* KCh 93.

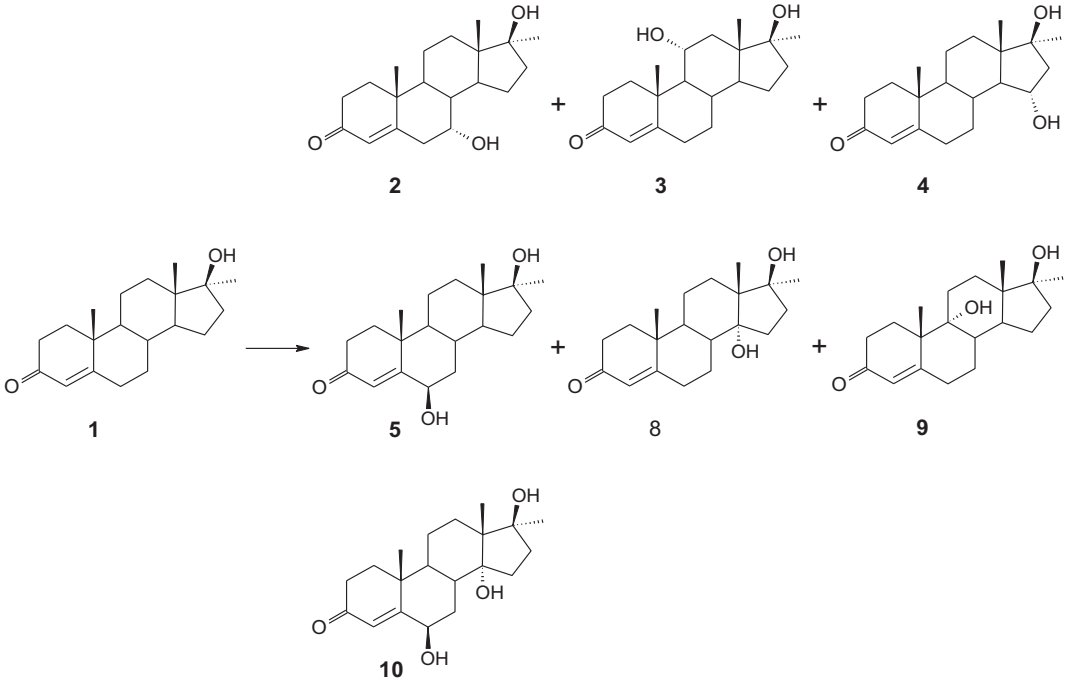


Fig. 3. Transformation of 17α-methyltestosterone (**1**) by *Chaetomium* sp. KCh 6651.

form compounds of diverse structure, such as cannabinoids [42], chalcones [43], and 8-prenylnaringenin [44]. Their ability to hydroxylate unsaturated lactones in the allylic position has also been demonstrated [45,46]. The literature also describes biotransformations of 3-hydroxysteroids – cinobufagin [47], diosgenin [48], protostane derivatives [49], rotundic [50] and ursolic acids [51,52] – for which several hydroxylated products were observed. Moreover, two new hydroxy-derivatives of 7-oxo-DHEA: 1β,3β-dihydroxy-androst-5-en-7,17-dione and 3β,12β-dihydroxy-androst-5-en-7,17-dione were obtained in the culture of this strain [53].

The biotransformation of 4-en-3-one steroids in *S. racemosum* cultures has not been described so far.

Absidia. coerulea strains have been described as highly selective in hydroxylation of steroid compounds, efficiently hydroxylated 16,17α-epoxyprogesterone leading to one product: 11β-hydroxy derivative [54]. DHEA in the culture of the same strain was transformed into 7α- and 7β-hydroxyderivatives [55]. A new CYP5311B1 11α-hydroxylase gene from *A. coerulea* AS3.65 [56], which may be responsible for hydroxylation at both positions 11α and 7, was identified [55]. The presence of similar enzyme was also confirmed by 7α-hydroxylation of 17α-methyltestosterone (**1**) [38]. In this work, we describe other hydroxylation products, in the 6β, 12β and 15α positions, what may indicate activity of different enzyme or the presence of other steroid monooxygenases in cells of *A. coerulea* KCh 93. This strain afforded five products (Fig. 2): the main product, 6β-hydroxy derivative (**5**), as well as 12β-, 7α-, 11α- and 15α-hydroxy derivatives, with yields of 44%, 29%, 6%, 5% and 9%, respectively.

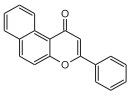
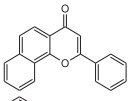
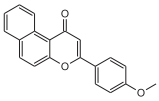
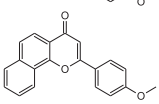
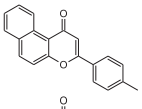
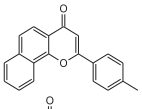
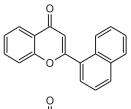
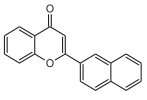
Chaetomium sp. KCh 6651 afforded five known products: **4**, **3**, **2**, **5** and **9** with yields of 31%, 20%, 16%, 7% and 5%, respectively. Moreover, two other products not described in the literature so far were obtained: 14α-hydroxy (**8**) with yield of 7% and 6β,14α-dihydroxy derivative (**10**) with a yield of 4% (Table 1 and Fig. 3). This result was a surprise because, in our previous biotransformation studies of other 4-en-3-oxo steroids, we obtained no more than three products. Testosterone was hydroxylated at the 7β position, androstenedione at the 14α position, while progesterone underwent double hydroxylation which resulted in 6β,14α-dihydroxy progesterone [57]. In addition to the previously described hydroxylations at C-6 and C-14 carbon atoms, 17α-methyltestosterone (**1**) also underwent functionalization at other non-activated carbon atoms of the steroid skeleton (C-7, C-9, C-11 and C-15).

Due to a large number of products resulting from the biotransformation of 17α-methyltestosterone (**1**) in cultures of tested strains and information that the medium's composition may affect the yields, we decided to determine whether a change in the source of both carbon and nitrogen will affect the composition of the products. Biotransformations were carried out using various sources of amino acids (aminobac, peptone and yeast extract) and various monosaccharides (D-ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, L-glucose, D-mannose and D-fructose) and disaccharides (lactose, maltose, sucrose). Based on this experiment, it was found that the change of these nutrient components does not affect the degree of substrate conversion and the composition of the products formed. Therefore, medium changes did not affect expression levels of monooxygenases, nor induced expression of new enzymes.

Table 2
Isolated products' yields obtained in transformation of 17α-methyltestosterone (**1**).

No. of compound	Isolated yields [%]		
	Chaetomium sp. KCh 6651	*A. coerulea* KCh 93	*S. racemosum* KCh 105
2	16	6	45
3	20	5	17
4	31	9	19
5	7	34	–
6	–	29	–
7	–	4	–
8	7	–	–
9	5	–	–
10	4	–	–

Table 3
Inhibitors used in the studies.

No. of compound	Structure of inhibitor	Recovery of the untransformed substrate (effect of added flavone) [%]		
		Chaetomium sp. KCh 6651	*A. coerulea* KCh 93	*S. racemosum* KCh 105
11		45 ± 5	40 ± 4	47 ± 6
12		47 ± 3	32 ± 1	49 ± 5
13		51 ± 6	45 ± 3	41 ± 5
14		49 ± 1	48 ± 2	45 ± 3
15		46 ± 6	42 ± 1	36 ± 4
16		34 ± 2	41 ± 5	38 ± 6
17		60 ± 3	73 ± 2	54 ± 4
18		49 ± 2	61 ± 3	51 ± 7

In the literature, numerous experiments on the use of α- and β-naphthoflavone (ANF and BNF) as inhibitors or inducers of monooxygenases have been described [58–61]. ANF has been described as an effective hydroxylation inhibitor at the 6β position of progesterone and testosterone catalysed by CYP3A6 in RIF-microsomes. Also, time- and concentration-dependent inactivation of human CYP3A4-mediated 6β-hydroxylation of testosterone by ANF was determined [62]. For this reason, it was decided to investigate the effect of selected derivatives of α- and β-naphthoflavone on monooxygenases of the investigated biocatalysts. The purpose of our experiment was to determine whether any of the tested naphthoflavones would be able to selectively modulate the activity of any of the monooxygenases involved in the methyltestosterone (**1**) transformation. The observed effect should be the change in the percentage composition of the obtained products. All applied α- and β-naphthoflavones (2 mg) were added one hour before addition of the substrate (20 mg) to the grown culture. A significant reduction in substrate conversion was observed. Table 2 shows the percentage of substrate present in the reaction mixture after incubation with tested naphthoflavones.

The contribution of the untransformed substrate was determined after three days of incubation of the substrate in the microbial cultures. The percentage of the substrate in the biotransformation without the addition of naphthoflavone did not exceed 1%. The strong inhibitors used in this study, α- and β-naphthoflavones, inhibited monooxygenases, and compound **15** was the most effective one (Table 3). A similar effect of ANF inhibiting P450 1A1 binding to benzo[*a*]pyrene via a classical competitive mechanism was described [63]. However, none of the used inhibitors changed the percentage composition of the obtained products. Since all monooxygenases catalysing the hydroxylation of steroid compounds have a very similar structure of the catalytic centre it is possible that inhibitors used were most likely cross-reacting with all active isoenzymes involved in the hydroxylation reactions of the test substrate. This conclusion is consistent with other research, where ANF cannot be regarded as specific for inhibition of CYP1A activity from either mammals or fish, even at low micromolar concentrations [58].

4. Conclusions

The selected microorganisms were characterised by high transformation ability of 17α-methyltestosterone (**1**).

All products were purified using chromatographic methods. By GC and TLC analyses as well as ^{1}H NMR, ^{13}C NMR and correlation spectroscopy, the structures of nine products were established, including two new ones not described in the literature so far. The compounds obtained through biotransformations can serve as standards of metabolites of higher organisms and contribute to understanding the metabolism of this important steroid in the human body.

Biotransformations were also carried out using various sources of nitrogen (aminobac, peptone, yeast extract) and carbon (D-ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, L-glucose, D-mannose, D-fructose, lactose, maltose, sucrose). It was found that the change of these nutrient components does not affect the degree of substrate conversion or the composition of the products formed.

The α- and β-naphthoflavones used as monooxygenase inhibitors or inducers resulted in inhibition of the hydroxylation process but did not change the percentage composition of the formed products. Due to this action, the tested naphthoflavones may applied in medicine as inhibitors of steroid compounds' metabolism for dose reduction of the drug.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bioorg.2018.03.021.

References

[1] L. You, Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes, Chem. Biol. Interact. 147 (2004) 233–246, https://doi.org/10.1016/j.cbi.2004.01.006.
[2] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Industrial biocatalysis today and tomorrow, Nature 409 (2001) 258–268, https://doi.org/10.1038/35051736.
[3] P. Žnidaršič-Plazl, I. Plazl, Development of a continuous steroid biotransformation process and product extraction within microchannel system, Catal. Today (2010) 315–320. http://doi.org/10.1016/j.cattod.2010.01.042.
[4] M.M. Chen, F.Q. Wang, L.C. Lin, K. Yao, D.Z. Wei, Characterization and application of fusidane antibiotic biosynethsis enzyme 3-ketosteroid-Δ^1-dehydrogenase in steroid transformation, Appl. Microbiol. Biotechnol. 96 (2012) 133–142, https://doi.org/10.1007/s00253-011-3855-5.
[5] M.V. Donova, O.V. Egorova, Microbial steroid transformations: current state and prospects, Appl. Microbiol. Biotechnol. 94 (2012) 1423–1447, https://doi.org/10.1007/s00253-012-4078-0.
[6] T. Kołek, N. Milecka, A. Świzdor, A. Panek, A. Białońska, Hydroxylation of DHEA, androstenediol and epiandrosterone by *Mortierella isabellina* AM212. Evidence indicating that both constitutive and inducible hydroxylases catalyze 7α- as well as 7β-hydroxylations of 5-ene substrates, Org. Biomol. Chem. 9 (2011) 5414–5422, https://doi.org/10.1039/c1ob05350g.
[7] T. Janeczko, N. Milecka, E. Kostrzewa-Susłow, Industrial importance of microbial hydroxylation of steroids, Przem. Chem. 91 (2012) 767–771.
[8] E. Kozłowska, M. Urbaniak, A. Kancelista, M. Dymarska, E. Kostrzewa-Susłow, Ł. Stępień, T. Janeczko, Biotransformation of dehydroepiandrosterone (DHEA) by environmental strains of filamentous fungi, RSC Adv. 7 (2017) 31493–31501, https://doi.org/10.1039/C7RA04608A.
[9] T. Janeczko, A. Świzdor, J. Dmochowska-Gładysz, A. Białońska, Z. Ciunik, E. Kostrzewa-Susłow, Novel metabolites of dehydroepiandrosterone and progesterone obtained in *Didymosphearia igniaria* KCH 6670 culture, J. Mol. Catal. B Enzym. 82 (2012) 24–31, https://doi.org/10.1016/j.molcatb.2012.05.009.
[10] K.B. Borges, W.de S. Borges, R. Durán-Patrón, M.T. Pupo, P.S. Bonato, I.G. Collado, Stereoselective biotransformations using fungi as biocatalysts, Tetrahedron: Asymmetry 20 (2009) 385–397, https://doi.org/10.1016/j.tetasy.2009.02.009.
[11] E. Kozłowska, M. Dymarska, E. Kostrzewa-Susłow, T. Janeczko, *Isaria fumosorosea* KCh J2 entomopathogenic strain as an effective biocatalyst for steroid compound transformations, Molecules 22 (2017) 1511, https://doi.org/10.3390/molecules22091511.
[12] O.J. Pozo, P. Van Eenoo, K. Deventer, L. Lootens, W. Van Thuyne, M.K. Parr, W. Schanzer, J.V. Sancho, F. Hernandez, P. Meuleman, G. Leroux-Roels, F.T. Delbeke, Detection and characterization of a new metabolite of 17-methyltestosterone, Drug Metab. Dispos. 37 (2009) 2153–2162, https://doi.org/10.1124/dmd.109.028373.
[13] W. Schänzer, M. Donike, Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites, Anal. Chim. Acta 275 (1993) 23–48, https://doi.org/10.1016/0003-2670(93)80274-O.
[14] S. Asha, M. Vidyavathi, *Cunninghamella* – a microbial model for drug metabolism studies – a review, Biotechnol. Adv. 27 (2009) 16–29, https://doi.org/10.1016/j.biotechadv.2008.07.005.
[15] S.M.R. Stanley, S. Kent, J.P. Rodgers, Biotransformation of 17-alkyl steroids in the equine: high-performance liquid chromatography-mass spectrometric and gas chromatography-mass spectrometric analysis of fluoxymesterone metabolites in urine samples, J. Chromatogr. B Biomed. Appl. 704 (1997) 119–128, https://doi.org/10.1016/S0378-4347(97)00440-4.
[16] J.F. Templeton, C.C. Jackson, Metabolism of 17α-methyltestosterone in the rabbit: C-6 and C-16 hydroxylated metabolites, Steroids 42 (1983) 115–121, https://doi.org/10.1016/S0039-128X(83)90144-7.
[17] M.C. Dumasia, In vivo biotransformation of 17 alpha-methyltestosterone in the horse revisited: identification of 17-hydroxymethyl metabolites in equine urine by capillary gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 320–329, https://doi.org/10.1002/rcm.909.
[18] G. Feist, C.G. Yeoh, M.S. Fitzpatrick, C.B. Schreck, The production of functional sex-reversed male rainbow trout with 17α-methyltestosterone and 11β-hydroxyandrostenedione, Aquaculture 131 (1995) 145–152, https://doi.org/10.1016/0044-8486(94)00336-M.

[19] C.L.G. Rivero-Wendt, R. Oliveira, M.S. Monteiro, I. Domingues, A.M.V.M. Soares, C.K. Grisolia, Steroid androgen 17α-methyltestosterone induces malformations and biochemical alterations in zebrafish embryos, Environ. Toxicol. Pharmacol. 44 (2016) 107–113, https://doi.org/10.1016/j.etap.2016.04.014.
[20] N. Mlalila, C. Mahika, L. Kalombo, H. Swai, A. Hilonga, Human food safety and environmental hazards associated with the use of methyltestosterone and other steroids in production of all-male tilapia, Environ. Sci. Pollut. Res. 22 (2015) 4922–4931, https://doi.org/10.1007/s11356-015-4133-3.
[21] G.T. Ankley, K.M. Jensen, M.D. Kahl, J.J. Korte, E.A. Makynen, Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*), Environ. Toxicol. Chem. 20 (2001) 1276–1290, https://doi.org/10.1897/1551-5028(2001) 020<1276:daeoas>2.0.co;2.
[22] I.J. Kang, H. Yokota, Y. Oshima, Y. Tsuruda, Y. Shimasaki, T. Honjo, The effects of methyltestosterone on the sexual development and reproduction of adult medaka (*Oryzias latipes*), Aquat. Toxicol. 87 (2008) 37–46, https://doi.org/10.1016/j.aquatox.2008.01.010.
[23] M. Seki, H. Yokota, H. Matsubara, M. Maeda, H. Tadokoro, K. Kobayashi, Fish full life-cycle testing for androgen methyltestosterone on medaka (*Oryzias latipes*), Environ. Toxicol. Chem. 23 (2004) 774–781, https://doi.org/10.1897/03-26.
[24] J. Popłoński, S. Sordon, T. Tronina, A. Bartmańska, E. Huszcza, Fungal metabolism of naphthoflavones, J. Mol. Catal. B Enzym. 117 (2015) 1–6, https://doi.org/10.1016/j.molcatb.2015.04.007.
[25] T. Janeczko, M. Dymarska, M. Siepka, R. Gniłka, A. Leśniak, J. Popłoński, E. Kostrzewa-Susłow, Enantioselective reduction of flavanone and oxidation of cis- and trans-flavan-4-ol by selected yeast cultures, J. Mol. Catal. B Enzym. 109 (2014) 47–52, https://doi.org/10.1016/j.molcatb.2014.08.006.
[26] N. Yadav, S.K. Dixit, A. Bhattacharya, L.C. Mishra, M. Sharma, S.K. Awasthi, V.K. Bhasin, Antimalarial activity of newly synthesized chalcone derivatives *in vitro*, Chem. Biol. Drug Des. 80 (2012) 340–347, https://doi.org/10.1111/j.1747-0285.2012.01383.x.
[27] B. Żyszka, M. Anioł, J. Lipok, Highly effective, regiospecific reduction of chalcone by cyanobacteria leads to the formation of dihydrochalcone: two steps towards natural sweetness, Microb. Cell Fact. 16 (2017) 136, https://doi.org/10.1186/s12934-017-0752-3.
[28] C. Dittmer, G. Raabe, L. Hintermann, Asymmetric cyclization of 2′-hydroxychalcones to flavanones: catalysis by chiral Brønsted acids and bases, Eur. J. Org. Chem. (2007) 5886–5898, https://doi.org/10.1002/ejoc.200700682.
[29] L.Y. Rok, K.K. Yong, Mild and efficient method for the synthesis of flavones by InCl3-mediated cyclization of 1,3- diketones: concise total synthesis of lanceolatin B, Let. Org. Chem. 4 (2007) 440–444.
[30] D. Hwang, G. Jo, J. Hyun, S.D. Lee, D. Koh, Y. Lim, Synthesis of methoxybenzoflavones and assignments of their NMR data, Magn. Reson. Chem. 50 (2012) 62–67, https://doi.org/10.1002/mrc.3790.
[31] A.L.F. Navarini, L.D. Chiaradia, A. Mascarello, M. Fritzen, R.J. Nunes, R.A. Yunes, T.B. Creczynski-Pasa, Hydroxychalcones induce apoptosis in B16–F10 melanoma cells via GSH and ATP depletion, Eur. J. Med. Chem. 44 (2009) 1630–1637, https://doi.org/10.1016/j.ejmech.2008.09.009.
[32] D. Hwang, J. Hyun, G. Jo, D. Koh, Y. Lim, Synthesis and complete assignment of NMR data of 20 chalcones, Magn. Reson. Chem. 49 (2011) 41–45, https://doi.org/10.1002/mrc.2707.
[33] M. Muthukumar, P. Viswanathamurthi, Ruthenium(II) chalconate complexes: synthesis, characterization, catalytic, and biological studies, Spectrochim. Acta – Part A Mol. Biomol. Spectrosc. 74 (2009) 454–462, https://doi.org/10.1016/j.saa.2009.06.041.
[34] D. Bennardi, D. Ruiz, G. Romanelli, G. Baronetti, H. Thomas, J. Autino, Efficient microwave solvent-free synthesis of flavones, chromones, coumarins and dihydrocoumarins, Lett. Org. Chem. 5 (2008) 607–615, https://doi.org/10.2174/157017808786857570.
[35] D.O. Bennardi, G.P. Romanelli, J.C. Autino, L.R. Pizzio, Trifluoromethanesulfonic acid supported on carbon used as catalysts in the synthesis of flavones and chromones, Catal. Commun. 10 (2009) 576–581, https://doi.org/10.1016/j.catcom.2008.10.040.
[36] D.N. Kirk, H.C. Toms, C. Douglas, K.a. White, K.E. Smith, S. Latif, R.W.P. Hubbard, A survey of the high-field 1H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds, J. Chem. Soc. Perkin Trans. 2 (1990) 1567, https://doi.org/10.1039/p29900001567.
[37] E. Huszcza, J. Dmochowska-Gładysz, A. Bartmańska, Transformations of steroids by *Beauveria bassiana*, Zeitschrift Fur Naturforsch. – Sect. C J. Biosci. 60 (2005) 103–108.
[38] E. Brzezowska, J. Dmochowska-Gładysz, T. Kołek, Biotransformation XXXIX. Metabolism of testosterone, androstenedione, progesterone and testosterone derivatives in *Absidia coerulea* culture, J. Steroid Biochem. Mol. Biol. 57 (1996) 357–362, https://doi.org/10.1016/0960-0760(95)00279-0.
[39] N.V. Carpova-Rodina, V.A. Andryushina, V.V. Yaderetz, A.V. Druzhinina, T.S. Stytsenko, B.L. Shaskol'skiy, V.I. Lozinsky, L.D. Huy, N.E. Voishvillo, Transformation of Δ4-3-ketosteroids by free and immobilized cells of *Rhodococcus erythropolis* actinobacterium, Appl. Biochem. Microbiol. 47 (2011) 386–392, https://doi.org/10.1134/S0003683811040041.
[40] S.P. Kolet, S. Niloferjahan, S. Haldar, R. Gonnade, H.V. Thulasiram, Biocatalyst mediated production of 6β,11α-dihydroxy derivatives of 4-ene-3-one steroids, Steroids 78 (2013) 1152–1158, https://doi.org/10.1016/j.steroids.2013.08.004.
[41] E. Huszcza, J. Dmochowska-Gladysz, Transformations of testosterone and related steroids in *Absidia glauca* culture, J. Basic Microbiol. 43 (2003) 113–120, https://doi.org/10.1002/jobm.200390011.
[42] L.W. Robertson, M.A. Lyle, S. Billets, Biotransformation of cannabinoids by *Syncephalastrum racemosum*, Biol. Mass Spectrom. 2 (1975) 266–271, https://doi.org/10.1002/bms.1200020505.
[43] T. Janeczko, W. Gładkowski, E. Kostrzewa-Susłow, Microbial transformations of chalcones to produce food sweetener derivatives, J. Mol. Catal. B Enzym. 98 (2013) 55–61, https://doi.org/10.1016/j.molcatb.2013.09.021.
[44] A. Bartmańska, T. Tronina, E. Huszcza, Microbial sulfation of 8-prenylnaringenin, Z. Naturforsch. C. 68 (2013) 231–235, https://doi.org/10.5560/ZNC.2013.68c0231.
[45] A. Grudniewska, C. Wawrzeńczyk, Lactones 41. Synthesis and microbial hydroxylation of unsaturated terpenoid lactones with p-menthane ring systems, Molecules 18 (2013) 2778–2787, https://doi.org/10.3390/molecules18032778.
[46] K. Wińska, M. Grabarczyk, W. Mączka, A. Kondas, G. Maciejewska, R. Bonikowski, M. Anioł, Synthesis and biotransformation of bicyclic unsaturated lactones with three or four methyl groups, Molecules 22 (2017) 147, https://doi.org/10.3390/molecules22010147.
[47] X. chi Ma, X. lan Xin, K. xin Liu, J. Han, D. an Guo, Microbial transformation of cinobufagin by *Syncephalastrum racemosum*, J. Nat. Prod. 71 (2008) 1268–1270, https://doi.org/10.1021/np800210a.
[48] Y. Zhao, L.M. Sun, X.N. Wang, T. Shen, M. Ji, X. Li, H.X. Lou, Microbial transformation of diosgenin by *Syncephalastrum racemosum* (Cohn) Schroeter, Chin. Chem. Lett. 21 (2010) 76–80, https://doi.org/10.1016/j.cclet.2009.08.015.
[49] C. Wang, X.K. Huo, B.J. Zhang, C.P. Sun, X.G. Tian, S. Deng, B. Li, W. Wang, P.P. Dong, X.C. Ma, Highly regioselective glucosylation of alcoholic hydroxyls of protostane triterpenoids mediated by fungal biotransformation, Catal. Commun. 89 (2017) 40–43, https://doi.org/10.1016/j.catcom.2016.10.025.
[50] H. Li, B. Yang, D. Cao, L. Zhou, Q. Wang, L. Rong, X. Zhou, J. Jin, Z. Zhao, Identification of rotundic acid metabolites after oral administration to rats and comparison with the biotransformation by *Syncephalastrum racemosum* AS 3.264, J. Pharm. Biomed. Anal. 150 (2018) 406–412, https://doi.org/10.1016/j.jpba.2017.12.026.
[51] S. Bin Fu, J.S. Yang, J.L. Cui, D.A. Sun, Biotransformation of ursolic acid by *Syncephalastrum racemosum* CGMCC 3.2500 and anti-HCV activity, Fitoterapia 86 (2013) 123–128, https://doi.org/10.1016/j.fitote.2013.02.007.
[52] F.X. Huang, W.Z. Yang, F. Ye, J.Y. Tian, H.B. Hu, L.M. Feng, D.A. Guo, M. Ye, Microbial transformation of ursolic acid by *Syncephalastrum racemosum* (Cohn) Schroter AS 3.264, Phytochemistry 82 (2012) 56–60, https://doi.org/10.1016/j.phytochem.2012.06.020.
[53] A. Świzdor, A. Panek, N. Milecka-Tronina, Biohydroxylation of 7-oxo-DHEA, a natural metabolite of DHEA, resulting in formation of new metabolites of potential pharmaceutical interest, Chem. Biol. Drug Des. 88 (2016) 844–849, https://doi.org/10.1111/cbdd.12813.
[54] K. Chen, W.-Y. Tong, D.-Z. Wei, W. Jiang, The 11β-hydroxylation of 16,17α-epoxyprogesterone and the purification of the 11β-hydroxylase from *Absidia coerulea* IBL02, Enzyme Microb. Technol. 41 (2007) 71–79, https://doi.org/10.1016/j.enzmictec.2006.12.002.
[55] N. Milecka-Tronina, T. Kołek, A. Świzdor, A. Panek, Hydroxylation of DHEA and its analogues by *Absidia coerulea* AM93. Can an inducible microbial hydroxylase catalyze 7α- and 7β-hydroxylation of 5-ene and 5α-dihydro C19-steroids?, Bioorg Med. Chem. 22 (2014) 883–891, https://doi.org/10.1016/j.bmc.2013.11.050.
[56] R. Wang, P. Sui, X. Hou, T. Cao, L. Jia, F. Lu, S. Singh, Z. Wang, X. Liu, Cloning and identification of a novel steroid 11α-hydroxylase gene from *Absidia coerulea*, J. Steroid Biochem. Mol. Biol. 171 (2017) 254–261, https://doi.org/10.1016/j.jsbmb.2017.04.006.
[57] T. Janeczko, J. Dmochowska-Gładysz, E. Kostrzewa-Susłow, A. Białońska, Z. Ciunik, Biotransformations of steroid compounds by *Chaetomium sp.* KCH 6651, Steroids 74 (2009) 657–661, https://doi.org/10.1016/j.steroids.2009.02.006.
[58] N. Takahashi, C.L. Miranda, M.C. Henderson, D.R. Buhler, D.E. Williams, G.S. Bailey, Inhibition of in vitro aflatoxin B1-DNA binding in rainbow trout by CYP1A inhibitors: α-naphthoflavone, β-naphthoflavone and trout CYP1A1 peptide antibody, Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 110 (1995) 273–280. http://doi.org/10.1016/0742-8413(95)00005-9.
[59] U. Kumari, N. Srivastava, A. Shelly, P. Khatri, N. Sarat, D.K. Singh, S. Mazumder, Inducible headkidney cytochrome P450 contributes to endosulfan immunotoxicity in walking catfish *Clarias gariepinus*, Aquat. Toxicol. 179 (2016) 44–54, https://doi.org/10.1016/j.aquatox.2016.08.009.
[60] T. Shimada, N. Murayama, K. Kakimoto, S. Takenaka, Y.R. Lim, S. Yeom, D. Kim, H. Yamazaki, F.P. Guengerich, M. Komori, Oxidation of 1-chloropyrene by human CYP1 family and CYP2A subfamily cytochrome P450 enzymes: catalytic roles of two CYP1B1 and five CYP2A13 allelic variants, Xenobiotica (2017) 1–11, https://doi.org/10.1080/00498254.2017.1347306.
[61] X. Meng, B. Zhigang Wang, B. Lianrong Yang, B. Shujun Xu, B. Yuan Tian, B. Hui Sun, Comparative α-naphthoflavone and β-naphthoflavone inhibits the formation of a carcinogenic estrogen metabolite, Int. J. Pept. Res. Ther. 23 (2017) 291–295, https://doi.org/10.1007/s10989-016-9560-6.
[62] L. Boek-Dohalská, P. Hodek, M. Šulc, M. Stiborová, α-Naphthoflavone acts as activator and reversible or irreversible inhibitor of rabbit microsomal CYP3A6, Chem. Biol. Interact. 138 (2001) 85–106, https://doi.org/10.1016/S0009-2797(01)00263-0.
[63] A.P. Koley, J.T.M. Buters, R.C. Robinson, A. Markowitz, F.K. Friedman, Differential mechanisms of cytochrome P450 inhibition and activation by α-naphthoflavone, J. Biol. Chem. 272 (1997) 3149–3152, https://doi.org/10.1074/jbc.272.6.3149.