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Microbial Baeyer–Villiger oxidation of steroidal ketones using *Beauveria bassiana*: Presence of an 11α-hydroxyl group essential to generation of D-homo lactones

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ABSTRACT

This paper demonstrates for the first time transformation of a series of 17-oxo steroidal substrates (epiandrosterone, dehydroepiandrosterone, androstenedione) by the most frequently used whole cell biocatalyst, *Beauveria bassiana*, to 11α-hydroxy-17a-oxa-D-homo-androst-17-one products, in the following sequence of reactions: 11α-hydroxylation and subsequent Baeyer–Villiger oxidation to a ring-D lactone. 11α-Hydroxyprogesterone, the product of the first stage of the progesterone metabolism, was further converted along two routes: hydroxylation to 6β,11α-dihydroxyprogesterone or 17β-acetyl chain degradation leading to 11α-hydroxytestosterone, the main metabolite of the substrate. Part of 11α-hydroxytestosterone underwent a rare reduction to 11α-hydroxy-5β-dihydrotestosterone. The experiments have demonstrated that the Baeyer–Villiger monooxygenase produced by the strain catalyzes solely oxidation of C-20 or C-17 ketones with 11α-hydroxyl group. 17-Oxo steroids, beside the 11α-hydroxylation and Baeyer–Villiger oxidation, also underwent reduction to 17β-alcohols; activity of 17β-hydroxysteroid dehydrogenase (17β-HSD) has significant impact on the amount of the formed ring-D δ-lactone.

1. Introduction

Baeyer–Villiger (BV) oxidation is a classic method of conversion of ketones into esters or lactones. The first report on enzymatic BV oxidation published over 50 years ago [1] reported conversion of progesterone to testololactone in *Penicillium* species and *Aspergillus flavus* by two sequential BV oxidation: Baeyer–Villiger degradation of 17β-acetyl side chain of progesterone to testosterone acetate, and ring D oxidation of androstenedione to testololactone. Further research has shown that the BV oxidation of various natural and xenobiotic ketones often constitutes the key metabolic stage of the carbon uptake route from carbonyl compounds in various microbial species [2,3]. After isolation of cyclohexanone monooxygenase from *Acinetobacter* sp. NCIB 9871 ($CHMO_{Acineto1}$) in 1976 [2], studies of the BV oxidation in degradation processes have evolved into intensive research on the reaction mechanism and the use of these specific enzymes in organic synthesis. Many microorganisms turned out to produce flavoenzymes capable of catalyzing BV oxidation, which were hereafter named "Baeyer–Villiger monooxygenases" (BVMO) [4]. Usually the BVMOs can convert compounds significantly different form their natural substrates, although substrate acceptance of the majority of enzymes carrying out BV oxidation of steroids is rather narrow—they are able to catalyze oxidation of steroidal substrates only.

Steroid BVMOs are substrate-induced enzymes [5–8], which mainly oxidize C-17 and/or C-20 carbonyl group in 4-en-3-oxo steroids. Literature reports from the last four years provide examples of BV oxidation of 5-en-3β-hydroxy steroidal ketones: DHEA and pregnenolone [9–12]. These substrates were converted according to two routes: BV oxidation to 3β-hydroxy-5-en ring-D lactone [9,11,12] or the BV oxidation was accompanied by transformation of 5-en-3β-hydroxy moiety to 4-en-3-ketone [10]; sometimes pregnenolone was not transformed because the microbial strain did not convert it into progesterone [10]. Transformations by the strain of *Aspergillus tamarii KITA* led, apart from the lactones, also to hydroxylactones: DHEA was transformed into ring-D lactone with conserved 5-en-3β-hydroxy moiety and its 7α-hydroxy derivative, while a mixture of 1β- and 11β-hydroxy-D-lactones was formed from 3α-hydroxy-5α-androstan-17-one [11,13]. In the culture of this strain, 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one was converted to a mixture of 1β-, 6β-, 7β-, 11α- and 11β-hydroxy derivatives [14]. Small amounts of 7α- and 9α-hydroxy derivatives were isolated after transformation of this 3β-hydroxy-D-lactone by *Cephalosporium aphidicola* [15]. 15α-Hydroxytestololactone together with the testololactone was among the products of DHEA transformation by *Penicillium griseopurpureum* Smith [16].

In the light of our interest [9,12] in the microbiological BV oxidation of steroids, we have endeavored to search for strains outside genus *Penicillium* and *Aspergillus* capable of producing steroidal lactones. This is important as it offers a potentially new route to

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novel biologically active steroids. Our screening studies have shown that the metabolic pathway of DHEA, androstenedione and progesterone in *Beauveria bassiana* KCH 1065 differed from other, reported in the literature, transformations of these substrates by various other strains of species *B. bassiana*—one of the most frequently used whole-cell biocatalysts [17,18]. The earlier literature reports on steroid transformation by *B. bassiana* provide mainly examples of regioselective 11α-hydroxylation of C-19 and C-21 4-en-3-oxo steroids [19–22]. In some transformations, degradation of the pregnane side chain was observed resulting in 11α-hydroxytestosterone [19,22].

We report here on the details of our investigation on metabolism of steroidal ketones by *B. bassiana* KCH 1065.

2. Materials and methods

2.1. Substrates

Epiandrosterone (3β-hydroxy-5α-androstan-17-one) (**1**), DHEA (dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one) (**2**), androstenediol (3β,17β-dihydroxyandrost-5-ene) (**3**), pregnenolone (3β-hydroxypregna-5-en-20-one) (**4**), androstenedione (4-androsten-3,17-dione) (**5**), and progesterone (4-pregnen-3,20-dione) (**6**) standards were purchased from Sigma-Aldrich Chemical Co. 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one was obtained in our laboratory by transformation of DHEA by *Penicillium lilacinum* AM111 [9]. It was found to be in excess of 99.2% purity following GC and elemental analysis, $C_{19}H_{28}O_3$: calcd. C, 74.96; H 9.27; found. C, 74.36; H, 9.19%; mp. 227–230 °C.

2.2. Microorganism

The organism used in the present study was isolated as a laboratory contaminant and identified as *B. bassiana* in the Department of Plant Protection, the Faculty of Life Sciences and Technology, Wrocław University of Environmental and Life Sciences, Poland. The identification was based on morphological features: shape of the colony, shape and size of conidiospores, color and size of hyphae, color and size of phialides [23,24]. The fungi were maintained on Sabouraud 4% dextrose-agar slopes at 4 °C and freshly subcultured before use in the transformation experiments.

2.3. Conditions of cultivation and transformation

General experimental and fermentation details have been described previously [9]. Substrate was added to 72-hours-old culture of the microorganism as an acetone solution, in a concentration of 0.08 mmol/100 ml of medium. Each experiment was performed with four replications.

2.4. Isolation and identification of metabolites

The fungal mycelium was separated from the broth by filtration under vacuum. The broth was then extracted three times with chloroform. The organic extract was dried over anhydrous magnesium sulfate and the solvent was subsequently evaporated *in vacuo* to give a brown gum. This gum was analyzed by TLC and GC and then chromatographed on a column of silica with the same eluent as for thin layer chromatography (see below). TLC was carried out with Merck Kieselgel 60 F_{254} plates with the use of the mixture of ethyl acetate/methylene chloride/acetone/isopropanol (3:1:0.5:0.15 vol./vol./vol.) as eluent. In order to develop the image, the plates were sprayed with solution of methanol in concentrated sulfuric acid (1:1 vol./vol.) and heated to 120 °C for 3 min. GC analysis was performed using Hewlett Packard 5890A Series II GC instrument (FID, carrier gas H_2 at flow rate of 2 ml min^{-1}) with an HP-1 column cross-linked methylsiloxane, 30 m × 0.53 mm × 1.5 μm film thickness. The applied temperature program was 220 °C/1 min, gradient 4 °C/min to 300 °C/3 min; injector and detector temperature was 300 °C. Retention times of the identified metabolites are given in Table 4. IR spectra were recorded in KBr disc on a Mattson IR 300 Spectrometer. The NMR spectra were measured in $CDCl_3$ or, when the solubility of metabolites in chloroform was low, either in CD_3OD or pyridine-d_5. The spectra were recorded on a DRX 300 MHz Bruker Avance spectrometer with TMS as internal standard. Characteristic shift values in the ^{1}H NMR and ^{13}C NMR spectra in comparison to the starting compounds were used to determine structures of metabolites, in combination with DEPT analysis to identify the nature of the carbon atoms (Tables 1, 2, and 3). The CD spectrum was recorded on a JASCO J-715 spectropolarimeter. Melting points (uncorrected) were determined on a Boetius apparatus. Elemental analysis was performed on vario EL III analyzer.

Table 1
^{13}C NMR data for starting materials **1**–**6** determined in $CDCl_3$, CD_3OD[a] or pyr-*d*[b].

Carbon atom	Compound					
	1([a])	**2**([a])	**3**([a])	**4**	**5**	**6**([b])
1	36.9 (38.2)	37.2 (38.5)	37.2 (38.6)	37.2	35.3	35.6 (35.8)
2	31.4 (32.1)	31.5 (32.8)	31.6 (32.7)	31.5	33.6	33.5 (35.3)
3	71.1 (71.8)	71.5 (72.3)	71.7 (72.4)	71.7	199.0	197.7 (198.2)
4	38.1 (38.9)	42.2 (43.0)	42.3 (43.0)	42.4	123.8	123.2 (123.2)
5	44.9 (46.3)	141.0 (142.5)	140.8 (142.4)	140.9	170.2	170.2 (170.2)
6	28.4 (29.7)	120.9 (122.0)	121.4 (122.3)	121.4	32.0	31.9 (34.3)
7	30.9 (32.1)	31.5 (32.7)	31.5 (32.3)	31.7	31.1	31.6 (32.6)
8	35.0 (36.4)	31.4 (32.3)	31.9 (33.4)	31.9	34.6	34.9 (35.3)
9	54.5 (55.9)	50.2 (51.9)	50.2 (51.9)	50.1	53.5	53.1 (53.6)
10	35.8 (36.8)	36.6 (37.9)	36.5 (37.8)	36.6	38.6	38.1 (31.2)
11	20.5 (21.6)	20.3 (21.5)	20.7 (21.9)	20.9	20.2	21.0 (21.1)
12	31.6 (32.9)	30.8 (31.9)	36.6 (37.9)	38.7	30.8	38.5 (38.6)
13	47.8 (49.1)	47.5 (48.2)	42.7 (43.9)	44.0	47.1	43.7 (43.8)
14	51.4 (52.8)	51.7 (53.1)	51.3 (52.7)	56.9	50.6	55.9 (55.8)
15	21.8 (22.8)	21.9 (22.9)	23.4 (24.4)	24.6	21.4	24.2 (24.4)
16	35.7 (36.7)	35.8 (36.7)	30.5 (30.7)	22.9	35.4	22.8 (23.0)
17	221.2 (224.1)	221.3 (223.8)	81.9 (82.5)	63.8	220.1	63.3 (63.3)
18	13.8 (14.2)	13.5 (14.0)	10.9 (11.6)	13.4	13.6	13.2 (13.3)
19	12.3 (12.7)	19.4 (19.9)	19.4 (20.0)	19.4	17.3	17.3 (17.0)
20				209.4		208.3 (208.2)
21				31.4		31.3 (32.0)

Table 2
^{13}C NMR data for metabolites **7**–**12** determined in $CDCl_3$ or CD_3OD[a].

Carbon atom	Compounds					
	7[a]	**8**[a]	**9**	**10**	**11**[a]	**12**[a]
1	39.8	39.9	38.4	39.0	40.4	40.4
2	32.4	32.5	30.6	31.7	33.5	35.5
3	71.5	71.6	70.7	71.7	72.6	72.5
4	39.5	39.5	38.8	42.6	43.6	43.3
5	46.6	46.7	44.5	141.6	142.9	142.7
6	30.3	30.5	28.6	120.7	122.1	121.4
7	32.0	32.9	31.7	31.4	32.5	32.3
8	35.6	36.4	36.9	30.8	32.8	32.2
9	61.3	61.3	59.3	57.0	57.9	56.9
10	38.5	38.4	37.2	38.3	39.5	39.5
11	69.2	69.5	68.7	68.7	69.5	69.0
12	43.8	49.4	49.9	42.7	54.8	50.3
13	48.2	44.5	81.2	47.9	44.5	83.6
14	51.7	51.4	45.5	50.7	51.8	47.0
15	22.8	24.3	20.0	21.8	24.2	20.8
16	36.7	30.8	28.7	35.9	30.8	29.4
17	222.5	82.1	170.7	219.0	82.0	174.4
18	14.9	12.6	21.0	14.3	12.4	21.1
19	13.0	13.1	12.4	19.1	19.5	19.2

2.5. Time course experiments

Time course experiments were conducted in order to determine the metabolic pathways. Conditions were identical to those in main biotransformation experiments. One flask was harvested after 3, 6, 9, 24 h, and every consecutive flask—another 24 h after transformation. Reaction mixtures were extracted and analyzed by GC and TLC as in section 2.4.

2.5.1. Transformation of DHEA (**2**) by substrate-induced culture of *B. bassiana*

After 63 h of fungal growth, 0.006 mmol of DHEA dissolved in 20 μl of acetone was added to the culture and incubation was continued under the same conditions (temperature, shaking) to those used for fungal growth. After another 9 h, 0.08 mmol of DHEA was added to the culture and transformation was continued for further 2 days. Every 24 h, the samples (5 ml) were taken, extracted and analyzed by GC as in section 2.4.

Table 3
^{13}C NMR data for metabolites **13**–**19** determined in $CDCl_3$ or pyr-d_5[a].

Carbon atom	Compounds					
	13	**14**	**15**	**16**	**17**[a]	**18**
1	37.1	37.6	37.5	36.9	38.7	40.0
2	33.9	34.2	34.0	34.0	35.0	38.5
3	198.9	199.8	200.1	199.9	200.2	214.1
4	124.5	124.3	124.6	124.3	126.4	42.7
5	170.1	170.8	169.6	171.0	170.3	46.0
6	33.2	33.4	33.0	33.4	72.6	25.2
7	31.4	31.6	29.9	31.6	39.7	26.8
8	33.9	34.5	37.3	34.9	28.9	34.8
9	59.8	59.9	58.0	58.8	59.6	47.3
10	40.1	40.5	39.7	39.7	39.9	36.0
11	68.8	69.0	68.5	68.5	68.3	68.6
12	42.4	47.8	49.7	50.2	50.7	48.5
13	47.9	43.2	81.4	44.2	44.3	43.5
14	49.7	50.1	45.1	55.1	55.7	50.0
15	21.6	23.4	19.9	24.3	24.4	23.3
16	35.4	30.6	28.3	30.0	23.1	30.6
17	219.2	80.9	171.0	63.1	63.3	81.2
18	14.5	12.1	21.1	14.3	14.5	12.1
19	18.1	18.0	18.1	18.2	20.5	23.1
20				208.0	207.9	
21				31.3	31.0	

2.5.2. Transformation of DHEA (**2**) by culture of *B. bassiana* with BVMO activity

DHEA (10 mg) dissolved in 0.25 ml of acetone was added to a 72-hours-old culture of the microorganism. After 9 h of transformation, further 10 mg of the substrate was added. The transformation conditions were the same as in the standard experiment. Twenty-four hours after addition of the first dose of DHEA, the reaction mixture was extracted and analyzed using GC.

2.5.3. Transformation of an equimolar mixture of androstenediol (**3**) and epiandrosterone (**1**) by *B. bassiana*

The transformation was carried out under standard conditions, using a mixture of 0.04 mmol of **3** and 0.04 mmol of **1** as a substrate.

2.6. Crystallographic structure determination of 6β,11α-dihydroxyprogesterone (**17**)

$C_{21}H_{30}O_4$, Mr = 346.45; monoclinic, space group $P2_1$, $a = 7.558(2)$, $b = 11.512(3)$, $c = 10.826(5)$ Å, $\beta = 96.74(3)°$, $V = 935.4(4)$ Å^3, $Z = 2$, $D_c = 1.230$ Mg m^{-3}, $\mu = 0.083$ mm^{-1}, $F(000) = 376$. X-ray data were collected using a colorless needle crystal of dimension $0.32 \times 0.15 \times 0.09$ mm^3 on a Kuma KM4CCD diffractometer (MoKα radiation; $\lambda = 0.71073$ Å). A total of 11,647 reflections were collected for $3.12 < \theta < 26.99°$ and $-9 \le h \le 9$, $-14 \le k \le 14$, $-13 \le l \le 13$. Data reduction and analysis were carried out with the *CrysAlis RED* program [25]. The space group was determined using the XPREP program. There were a total of 2145 independent reflections and 1458 reflections with $I > 2\sigma(I)$ used in the refinement. No absorption correction was applied. The structure was solved by direct methods using the XS program and refined using all F^2 data, as implemented by the XL program [26]. Non-hydrogen atoms were refined with anisotropic displacement parameters. The H atoms were found in $\Delta\rho$ map or placed at calculated positions. Before the last cycle of refinement all H atoms were fixed and were allowed to ride on their parent atoms. The Friedel pairs were merged before the final refinement. The final R indices were ($I > 2\sigma(I)$) $R_1 = 0.044$, $wR_2 = 0.080$ and R indices (all data) $R_1 = 0.078$, $wR_2 = 0.090$. The goodness of fit on F^2 was 1.014 and the largest difference peak and hole were 0.16 and −0.13 e Å^{-3}. The data for this structure were deposited in the Cambridge Crystallographic Data Centre under the number 785066 and can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +(44) 1223-336-033; e-mail, deposit@ccdc.cam.ac.uk.

3. Results

3.1. Products isolated in the course of transformations

3.1.1. Transformation of epiandrosterone (**1**)

After 48 h transformation of 100 mg of epiandrosterone (**1**) the following compounds were isolated (% mol): 3β,11α,17β-trihydroxy-5α-androstane (**8**) (14 mg, 13%) and 3β,11α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**) (69 mg, 62%). After 6 h transformation of 100 mg of 1 the additional isolate was 12 mg (11%) of 3β,11α-dihydroxy-5α-androstan-17-one (**7**).

3β,11α-Dihydroxy-5α-androstan-17-one (**7**): mp. 102–104 ° C (Me_2CO, plates) (lit. 103–106° [27]); $C_{19}H_{30}O_3$: calcd. C, 74.47; H, 9.87; found. C, 74.51; H, 9.83%. IR ν_{max} (cm^{-1}): 3470, 1741, ^{1}H NMR (CD_3OD) 0.91 (3H, s, 18-H), 1.02 (3H, s, 19-H), 3.56 (1 H, m, 3α-H), 3.95 (1H, d*t*, $J = 5.1$ Hz, $J = 10.5$ Hz, 11β-H).

3β,11α,17β-Trihydroxy-5α-androstane (**8**): mp. 241–245 ° C (Me_2CO, needles) (lit. 245–248° [28]); $C_{19}H_{32}O_3$: calcd. C, 73.98; H, 10.46; found. C, 74.10; H, 10.42%. R ν_{max} (cm^{-1}): 3318, ^{1}H NMR (CD_3OD) 0.77 (3H, s, 18-H), 1.00 (3H, s, 19-H), 3.53 (1H, m, 3α-H),

3.62 (1H, *t*, $J=8.5$ Hz, 17α-H), 3.91 (1H, d*t*, $J=5.1$ Hz, $J=10.5$ Hz, 11β-H).

3β,11α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**): mp. 257–258 ° C (Me_2CO, needles) (lit. 260° [14]); $C_{19}H_{30}O_4$: calcd. C, 70.77; H, 9.38; found. C, 70.70; H, 9.35%. IR ν_{max} (cm^{-1}): 3443, 1719, ^{1}H NMR ($CDCl_3$) 0.90 (3H, s, 19-H), 1.28 (3H, s, 18-H), 3.59 (1H, m, 3α-H), 3.75 (1H, d*t*, $J=4.8$ Hz, $J=11.4$ Hz, 11β-H).

3.1.2. Transformation of DHEA (2)

After 48 h transformation of 100 mg of DHEA (**2**), there were isolated (% mol): 3β,11α,17β-trihydroxyandrost-5-ene (**11**) (48 mg, 45%) and 3β,11α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**) (29 mg, 27%). After 3 h transformation of 100 mg of **2**, the additional isolates were 20 mg (20%) of androstenediol (**3**) and 7 mg (7%) of 11α-hydroxy-DHEA (**10**).

Androstenediol (**3**) IR ν_{max} (cm^{-1}): 3423, 3210, 1651, ^{1}H NMR ($CDCl_3$) 0.76 (3H, s, 18-H), 1.02 (3H, s, 19-H), 3.54 (1H, m, 3α-H), 3.64 (1H, *t*, $J=8.5$ Hz, 17α-H), 5.35 (1H, d, $J=5.1$ Hz, 6-H).

11α-Hydroxy-DHEA (**10**): mp. 210–212 ° C (Me_2CO, needles) (lit. 210° [29]); $C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27; found. C, 74.99; H, 9.25%. IR ν_{max} (cm^{-1}): 3446, 1739, 1654, ^{1}H NMR ($CDCl_3$) 0.90 (3H, s, 18-H), 1.18 (3H, s, 19-H), 3.53 (1H, m, 3α-H), 4.09 (1H, d*t*, $J=5.1$ Hz, $J=11.1$ Hz, 11β-H), 5.43 (1H, d, $J=5.2$ Hz, 6-H).

3β,11α,17β-Trihydroxyandrost-5-ene (**11**): mp. 261–265 ° C (MeOH, plates) (lit. 265–267° [30]); $C_{19}H_{30}O_3$: calcd. C, 74.47; H, 9.87; found. C, 74.50; H, 9.84%. IR ν_{max} (cm^{-1}): 3398, 3222, 1640, ^{1}H NMR (CD_3OD) 0.81 (3H, s, 18-H), 1.21 (3H, s, 19-H), 3.42 (1H, m, 3α-H), 3.65 (1H, *t*, $J=8.5$ Hz, 17α-H), 4.06 (1H, d*t*, $J=4.8$ Hz, $J=11.0$ Hz, 11β-H), 5.44 (1H, d, $J=4.9$ Hz, 6-H).

3β,11α-Dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**): mp. 234–236 ° C (MeOH, needles colorless). $C_{19}H_{28}O_4$: calcd. C, 71.22; H, 8.81; found. C, 71.15; H, 8.78%. IR ν_{max} (cm^{-1}): 3440, 1716, 1622, ^{1}H NMR (CD_3OD) 1.20 (3H, s, 19-H), 1.38 (3H, s, 18-H), 3.45 (1H, m, 3α-H), 3.88 (1H, d*t*, $J=4.8$ Hz, $J=11.0$ Hz, 11β-H), 5.45 (1H, d, $J=5.6$ Hz, 6-H).

3.1.3. Transformation of androstenediol (3)

After 4 days of transformation of 100 mg of androstenediol (**3**), the following compounds were isolated (% mol): the unreacted substrate (9 mg, 9%), 3β,11α,17β-trihydroxyandrost-5-ene (**11**) (22 mg, 21%) and 3β,11α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**) (63 mg, 58%).

3.1.4. Transformation of pregnenolone (4)

Transformation of pregnonolone (**4**) with the fungus for 6 days resulted in no observable transformation with full recovery of the starting material. It was identified by comparison of the NMR data with that of an authentic sample.

3.1.5. Transformation of androstenedione (AD) (5)

After 48 h of transformation of 100 mg of androstenedione (**5**), the isolates were (% mol) 8 mg (8%) of 11α-hydroxy-AD (**13**), 42 mg (40%) of 11α-hydroxytestosterone (**14**), and 27 mg (25%) of 11α-hydroxy-17a-oxa-D-homo-androst-4-en-3,17-dione (**15**).

11α-Hydroxy-AD (**13**): mp. 196 ° C (MeOH, cubes) (lit. 196–197° [20]); $C_{19}H_{26}O_3$: calcd. C, 75.46; H, 8.67; found. C, 75.37; H, 8.64%. IR ν_{max} (cm^{-1}): 3412, 1740, 1654, 1621, ^{1}H NMR ($CDCl_3$) 0.96 (3H, s, 18-H), 1.36 (3H, s, 19-H), 4.09 (1H, d*t*, $J=5.1$ Hz, $J=10.1$ Hz, 11β-H), 5.77 (1H, s, 4-H).

11α-Hydroxytestosterone (**14**): mp. 221 ° C (MeOH, cubes) (lit. 218–219° [20]); $C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27; found. C, 74.85; H, 9.25%. IR ν_{max} (cm^{-1}): 3420, 1660, 1618, ^{1}H NMR ($CDCl_3$) 0.80 (3H, s, 18-H), 1.30 (3H, s, 19-H), 3.66 (1H, *t*, $J=8.5$ Hz, 17α-H), 4.02 (1H, d*t*, $J=5.0$ Hz, $J=10.1$ Hz, 11β-H), 5.71 (1H, s, 4-H).

11α-Hydroxy-17a-oxa-D-homo-androst-4-en-3,17-dione (**15**): mp. 102–103 °C (chloroform, plates); $C_{19}H_{26}O_4$: calcd. C, 71.67; H, 8.23; found. C, 71.59; H, 8.21%. IR ν_{max} (cm^{-1}): 3370, 1719, 1654, ^{1}H NMR ($CDCl_3$) 1.29 (3H, s, 19-H), 1.35 (3H, s, 18-H), 3.84 (1H, d*t*, $J=4.5$ Hz, $J=10.1$ Hz, 11β-H), 5.73 (1H, s, 4-H).

3.1.6. Transformation of progesterone (6)

After 24 h of transformation of 100 mg of progesterone (**6**), the isolates were (% mol): the unreacted substrate (9 mg, 9%), 4 mg (4%) of 11α-hydroxyprogesterone (**16**), 14 mg (13%) of 6β,11α-dihydroxyprogesterone (**17**), 19 mg (20%) of 11α-hydroxytestosterone (**14**), and 19 mg (20%) of 11α,17β-dihydroxy-5β-androstan-3-one (**18**).

11α-Hydroxyprogesterone (**16**): mp. 169 °C (MeOH, needles) (lit. 165–168° [31]); $C_{21}H_{30}O_3$: calcd. C, 76.33; H, 9.15; found. C, 76.27; H, 9.12%. IR ν_{max} (cm^{-1}): 3454, 1695, 1656, 1620, ^{1}H NMR ($CDCl_3$) 0.70 (3H, s, 18-H), 1.32 (3H, s, 19-H), 2.14 (3H, s, 21-H), 4.05 (1H, d*t*, $J=4.8$ Hz, $J=10.5$ Hz, 11β-H), 5.74 (1H, s, 4-H).

6β,11α-Dihydroxyprogesterone (**17**): mp. 246–247 ° C (MeOH, needles) (lit. 244–246° [31]); $C_{21}H_{30}O_4$: calcd. C, 72.80; H, 8.73; found. C, 72.15; H, 8.71%. IR ν_{max} (cm^{-1}): 3444, 3378, 1696, 1658, ^{1}H NMR (pyr-d_5) 0.74 (3H, s, 18-H), 1.81 (3H, s, 19-H), 2.05 (3H, s, 21-H), 4.35 (1H, d*t*, $J=4.5$ Hz, $J=10.2$ Hz, 11β-H), 4.55 (1H, br s, 6α-H), 6.08 (1 H, s, 4-H).

11α,17β-Dihydroxy-5β-androstan-3-one (**18**): mp. 212–216 °C (MeOH, prisms); $C_{19}H_{30}O_3$: calcd. C, 74.47; H, 9.87; found. C, 74.20; H, 9.84%. IR ν_{max} (cm^{-1}): 3478, 1709, ^{1}H NMR ($CDCl_3$) 0.77 (3H, s, 18-H), 1.14 (3H, s, 19-H), 3.70 (1H, *t*, $J=8.5$ Hz, 17α-H), 4.00 (1H, m, 11β-H). CD ($c=1.1\times10^{-3}$ mol/l in methanol) $\Delta\varepsilon_{269}-0.70$.

3.2. Structural identification of metabolites

Incubation of epiandrosterone (**1**) resulted in isolation of three metabolites. The first product had a resonance at δ_H 3.56 ppm (m), confirming that the 3β-OH group was maintained. This product also had new resonances at δ_C 69.2 ppm and δ_H 3.95 ppm (d*t*, $J=5.1$ Hz, $J=10.5$ Hz), which suggested hydroxylation at an equatorial position of the steroid molecule. In comparison to the spectrum of substrate, the C-19 methyl signal of the product demonstrated significant downfield shift indicating that hydroxylation had occurred at the 11α-position. This was further supported by the ^{13}C NMR of **7** which showed downfield shifts for β-carbons C-9 (Δ 5.4 ppm) and C-12 (Δ 10.9 ppm) that were consistent with reference shifts values [32]. All these results confirmed that the product was 3β,11α-dihydroxy-5α-androstan-17-one (**7**). The spectroscopic data of this compound correspond to those described in literature [29]. The ^{1}H NMR spectrum of **8** possessed a resonance at δ_H 3.53 ppm (m), confirming that the 3β-hydroxy-5α-androstan skeleton had been retained. In comparison to the spectrum of **1**, it contained two new signals at δ_H 3.62 ppm (*t*, $J=8.5$ Hz) and δ_H 3.91 ppm (d*t*, $J=5.1$ Hz, $J=10.5$ Hz) thus suggesting the presence of two hydroxyl groups. The upfield shift of the C-18 methyl group (Δ 0.14 ppm) in ^{1}H NMR and the loss of the non-protonated resonance signal at δ_C 224.1 ppm, coupled with the appearance of a new methine carbon signal at δ_C 82.1 ppm in the ^{13}C NMR spectrum, confirmed that the C-17 ketone had been reduced to a C-17β alcohol. Further evidence of the reduction was provided by upfield shifts of C-13 (Δ 4.6 ppm) and C-16 (Δ 5.9 ppm) signals of this metabolite in comparison to the spectrum of substrate **1**. The site and the stereochemistry of the additional hydroxyl group were established from changes (β-deshielding effects) in the ^{13}C NMR spectra and the characteristic shape and multiplicity of the C*H*(OH) resonance in the ^{1}H NMR. All these results fully confirmed the structure of metabolite **8** as 3β,11α,17β-trihydroxy-5α-androstane. Comparison of ^{1}H NMR spectrum of the product **9** to that of epiandrosterone (**1**) revealed a new signal at δ_H 3.75 (d*t*, $J=4.8$ Hz, $J=11.4$ Hz), indicating monohydroxylation. The characteristic shape and multiplicity of this signal suggested that the hydroxyl group was introduced at 11α-position. It was confirmed by the long-range correlation of the

proton signal at δ_H 3.75 ppm with C-9 (δ_C 59.3 ppm), C-12 (δ_C 49.9 ppm), C-8 (δ_C 36.9 ppm) and C-10 (δ_C 37.2 ppm) carbon signals in HMBC spectrum (Fig. 1) and by downfield shift for C-9 (Δ 4.8 ppm) signal. Additionally, ROESY spectrum showed correlation of 11-Hβ with the proton signals of C-18 and C-19 methyl groups (Fig. 1). Lack of the signal of the C-17 at δ_C 221.2 ppm, emergence of a new signal at δ_C 170.7 ppm and downfield shift (Δ 33.4 ppm) for the C-13 resonance with respect to the starting compound **1** in the ^{13}C NMR spectrum, suggested oxygen insertion into ring-D and thereby formation of D lactone. It was supported by a significant downfield shift (Δ 0.46 ppm) for the 18-methyl protons' signal. Finally, that metabolite was identified as 3β,11α-dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**). Its ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature [14].

Incubation of DHEA (**2**) with *B. bassiana* KCH 1065 gave four metabolites which were separated by chromatography on silica. The first metabolite was readily identified as 3β,17β-dihydroxyandrost-5-ene (**3**) by comparison of the NMR data with that of an authentic sample. The structure of 11α-hydroxy-DHEA (**10**) was determined as a result of comparison of its spectroscopic data with that of the starting material **2**. A new signal at δ_H 4.09 ppm (d*t*, J = 5.1 Hz, J = 11.1 Hz) was observed in ^{1}H NMR spectrum, which suggested monohydroxylation. This observation was supported by appearance of a new methine carbon signal at δ_C 68.7 ppm which, in combination with the downfield shift of the 19-methyl protons signal (Δ 0.13 ppm) and downfield shift for C-9 (Δ 6.8 ppm) and C-12 (Δ 11.9 ppm) resonances, was an important confirmation of 11α-hydroxylation. The main metabolite, 3β,11α,17β-trihydroxyandrost-5-ene (**11**), was readily identified by comparison of its NMR data to that of compound **3**. ^{1}H NMR spectrum of **11** had a new resonance signal at δ_H 4.06 ppm (d*t*) consistent with substitution at an equatorial proton. Downfield β-carbon shifts in the ^{13}C NMR spectra for C-9 (Δ 6.0 ppm) and C-12 (Δ 16.9 ppm) confirmed hydroxylation at C-11. In the NMR spectra of product **12**, the presence of two C*H*(OH) resonances (δ_H 3.45 ppm and δ_H 3.88 ppm) and two oxygen-bearing methine carbon signals (δ_C 72.5 ppm and δ_C 69.0 ppm) suggested that the hydroxyl groups were at C-3 and C-11 positions. In comparison to the ^{1}H NMR spectrum of 11α-hydroxy-DHEA (**10**), the C-18 methyl protons signal had undergone a significant downfield shift (Δ 0.48 ppm) suggesting oxygen atom insertion near that group. The 5-ring ketone signal in **10** at δ_C 219 ppm was replaced by a non-protonated resonance at δ_C 174.4 ppm in the ^{13}C NMR spectrum of **12**, and this was consistent with D-ring extension. The 17a position of oxygen insertion was confirmed by significant downfield shifts of C-13 (Δ 35.7 ppm) and C-18 methyl (Δ 6.8 ppm) signals. X-ray crystallography [33] fully confirmed hydroxylation with 11α stereochemistry and D-ring lactonization and finally the structure of compound **12** as 3β,11α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one.

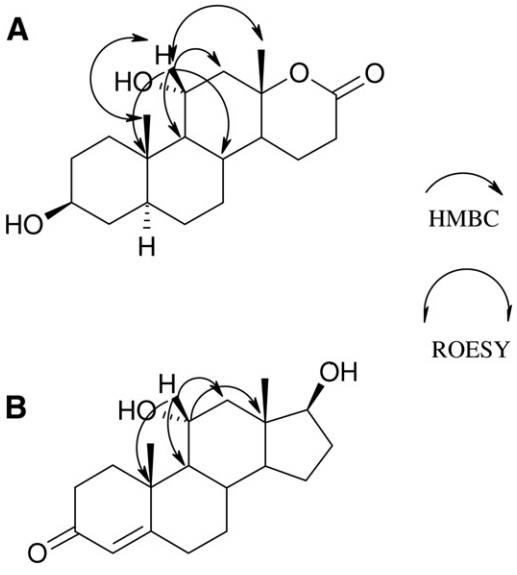

Fig. 1. Selected 2D NMR correlations of metabolite **9** (A) and **14** (B).

Incubation of androstenedione (**5**) generated three products of metabolism. Product **13** turned out to be a monohydroxylated androstenedione. Its NMR spectra showed a new downfield signal for the oxygen-bearing methine proton at δ_H 4.09 ppm (d*t*, J = 5.1 Hz, J = 10.1 Hz) and δ_C 68.8 ppm, which indicated introduction of a C-11α hydroxyl group. It was supported by the significant downfield shifts for C-9 (Δ 6.3 ppm) and C-12 (Δ 11.6 ppm) signals and other downfield shifts for C-1 (Δ 1.8 ppm) and C-10 (Δ 1.5 ppm), which were observed in ^{13}C NMR spectra. The spectral data for **13** are in agreement with those reported in the literature [20]. The NMR spectral data of compound **14** strongly suggested the presence of two hydroxyl groups. In the ^{13}C NMR spectrum, the characteristic absorption for the 17-carbonyl disappeared and a signal was found at δ_C 80.9 ppm instead of δ_C 220.1 ppm as compared to the parent compound **5**, which confirmed that it was reduced. The additional signal at δ_C 69.0 ppm was related to C-11. Its connected proton was designated to δ_H 4.02 ppm by HSQC experiment. The chemical shifts and multiplicity of H-17 (*t*) and H-11 (d*t*) suggested β- and α-stereochemistry of hydroxyl groups, respectively. The HMBC correlations of H-11 with C-9, C-10, C-12 and C-13 supported hydroxylation at C-11 (Fig. 1). Further evidence for 11α-hydroxylation and for the presence of 17β-OH group was provided by downfield shifts of C-9 (Δ 6.4 ppm) and C-12 (Δ 17.0 ppm), and upfield shifts of C-13 (Δ 3.9 ppm) and C-16 (Δ 4.8 ppm) signals. All the above is a proof that metabolite **14** is 11α-hydroxytestosterone. 11α-Hydroxy-17a-oxa-D-homo-androst-4-en-3,17-dione (**15**) was identified by the characteristic shape and multiplicity of C*H*(OH) signal at δ_H 3.84 ppm (d*t*, J = 4.5 Hz, J = 10.1 Hz), thereby suggesting introduction of the hydroxyl group at 11α-position. This was supported by a downfield shift of C-9 (Δ 4.5 ppm) signal in the ^{13}C NMR spectrum. The loss of signal at δ_C 220.1 ppm assigned to C-17 in the ^{13}C NMR spectrum of the starting material **5** and its replacement with a signal at δ_C 171.0 ppm together with a downfield shift of the C-13 resonance signal from δ_C 47.1 ppm to δ_C 81.4 ppm were consistent with lactone formation. This was coupled with the significant upfield shift (Δ 0.42 ppm) of the 18-methyl resonance signal. Thus, the shifts of signals in NMR spectra of **15** were consistent with 11α-hydroxylation and the formation of a ring D lactone, as in **9** and **12**.

Transformation of progesterone (**6**) yielded four products. Compound **16** was an 11α-hydroxylated derivative of progesterone. Its NMR spectra contained a new resonance signal at δ_H 4.05 ppm (d*t*) with coupling constants characteristic for 11α-hydroxylation. The disappearance of C-11 signal at δ_C 21.0 ppm (when compared to that of **6**), presence of a new oxygen-bearing methine signal at δ_C 68.5 ppm, and downfield shifts for C-9 (Δ 5.7 ppm) and C-12 (Δ 11.7 ppm) fully confirmed hydroxylation at C-11 position. The NMR data obtained are in agreement with those reported in the literature [34]. The ^{1}H NMR spectrum of metabolite **17** exhibited the presence of two C*H*(OH) signals at δ_H 4.35 ppm and δ_H 4.55 ppm. Position and shape (d*t*) of the first one can easily be assigned to 11β-H. The resonance signal at δ_H 4.55 ppm (br s) was consistent with substitution at an axial proton. The significant downfield shift of C-19 methyl protons signal (Δ 0.49 ppm) in comparison to the spectrum of **16** suggested 6β-hydroxylation. A shift for 4-H (6.08 ppm) also supported this assignment. The proposed structure was further confirmed by ^{13}C NMR in which two methylene signals were replaced by two methine signals, and β-carbon downfield shifts for C-7 (Δ 7.1 ppm), C-9 (Δ 6.0 ppm) and C-12 (Δ 12.1 ppm), and γ-carbon upfield shift for C-8 (which is in γ position to both C-6 and C-11, Δ 6.4 ppm) were observed. The signals appearing at δ_C 68.3 ppm and δ_C 72.6 ppm were assigned to C-11 and C-6 respectively, by the

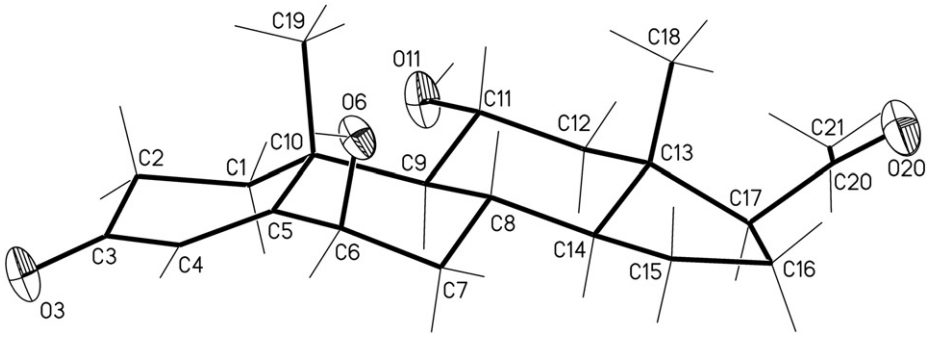

Fig. 2. X-ray crystal structure of 6β,11α-dihydroxyprogesterone (**17**). Hydrogen atoms are omitted for clarity.

assistance of HSQC experiment. The structure of compound **17** was unambiguously proved by X-ray structural analysis. A perspective view of the molecule is shown in Fig. 2. The NMR spectra of metabolite **18** revealed that there were two methyl groups, two hydroxyl groups and no carbon-carbon double bond. In comparison to the ^{13}C NMR spectrum of **14**, the disappearance of the double bond resonance signals at δ_C 170.8 ppm and δ_C 124.3 ppm confirmed hydrogenation of the double bond. This was supported by a new presence of methylene C-4 resonance signal at δ_C 40.0 ppm and methine C-5 resonance signal at δ_C 46.0 ppm. Hydrogenation resulting in 5β-stereochemistry at A/B ring was determined by chemical shift of C-19 methyl group signal, which was resonating at similar field (δ_C 23.1 ppm) when compared with a known 5β-isomer [32], and chemical shift of C-9 signal at higher field when compared with position of the analogous signal in the ^{13}C NMR spectrum of **8**. Confirmation of 5β-dihydrogenation was achieved by an n.O.e experiment where irradiation of the C-19 methyl hydrogens, enhanced the multiplets at δ_H 1.78–1.80 ppm, at δ_H 1.37–1.42 ppm and at δ_H 2.73–2.76 ppm assigned respectively to the C-5, C-1β and C-1α hydrogens. Additional evidence was provided by circular dichroism spectrum of this product, in which the negative Cotton effect (CE) was observed. A negative CE is characteristic of the *cis*-linkage of rings A/B in 3-oxo-5β-steroids [35]. Therefore, the metabolite **18** was proposed to be 11α,17β-dihydroxy-5β-androstan-3-one.

3.3. Determination of the metabolic pathway and the order of hydroxylation and lactonization reactions

In order to investigate metabolic pathways of the substrates **1**–**6**, composition of mixtures sampled after various transformation periods was studied. The results are compiled in Table 4. Their analysis indicates that the first stage of the process was 11α-hydroxylation of the substrates. The resulting 11α-hydroxy-17-oxo derivatives were further metabolized through BV oxidation (to 11α-hydroxy-D-lactones **9**, **12**, **15**); some of these derivatives were also reduced to 17β-alcohols. In all cases of transformations, the 11α-hydroxy derivatives were detected earlier than the products of BV oxidation. 11α-Hydroxyprogesterone, formed during the first stage of progesterone (**6**) transformation, was further transformed via two routes: through 6β-hydroxylation or the 17β-side chain cleavage leading to 11α-hydroxytestosterone (**14**)—its main metabolite. A fraction of 11α-hydroxytestosterone was subsequently reduced to 11α-hydroxy-5β-dihydrotestosterone (**18**). Pregnenolone was not susceptible to 11α-hydroxylation and was not metabolized at all (Figs. 3 and 4).

The obtained results (Table 4) indicated that enzymes catalyzing oxidation reactions are inducible. The reaction mixture, after 3 h incubation of epiandrosterone (**1**), contained 3% of 11α-hydroxyepiandrosterone (**7**), and after further 3 h, the share of 11α-hydroxy

Table 4
Composition of crude mixtures obtained in transformations of **1**–**6** by *Beauveria bassiana*.

Substrate	R_t (min)	Compounds present in the mixture (%)[a]	Time of transformation					
			3 (h)	6 (h)	9 (h)	24 (h)	48 (h)	96 (h)
Epiandrosterone (**1**)	3.98	Epiandrosterone (**1**)	96	48	5	–	–	
	6.06	3β,11α-Dihydroxy-5α-androstan-17-one (**7**)	3	15	10	–	–	
	6.43	3β,11α,17β-Trihydroxy-5α-androstane (**8**)	–	31	60	31	20	
	11.22	3β,11α-Dihydroxy-17a-oxa-D-homo-5α-androstan-17-one (**9**)	–	3	14	60	75	
DHEA (**2**)	3.46	DHEA (**2**)	51	2	3	–	–	
	3.79	Androstenediol (**3**)	24	2	–	–	–	
	5.11	11α-Dydroxy-DHEA (**10**)	9	6	4	3	4	
	5.34	3β,11α,17β-Trihydroxyandrost-5-ene (**11**)	15	87	77	65	56	
	8.54	3β,11α-Dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**)	–	–	10	22	34	
Androstenediol (**3**)	3.79	Androstenediol (**3**)	100	100	97	76	52	11
	5.34	3β,11α,17β-Trihydroxyandrost-5-ene (**11**)	–	–	3	22	41	25
	8.54	3β,11α-Dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**)	–	–	–	–	4	64
Pregnenolone (**4**)	5.33	Pregnenolone (**4**)	100	100	100	100	96	94
Androstenedione (**5**)	4.13	Androstenedione (**5**)	97	83	68	–	–	
	6.34	11α-Dydroxy-AD (**13**)	1	12	10	6	8	
	7.19	11α-Hydroxytestosterone (**14**)	–	3	17	57	42	
	10.35	11α-Hydroxy-17a-oxa-D-homo-androst-4-en-3,17-dione (**15**)	–	–	2	14	26	
Progesterone (**6**)	6.56	Progesterone (**6**)	81	21	17	12	10	
	8.90	11α-Hydroxyprogesterone (**16**)	9	3	2	7	1	
	10.85	6β,11α-Dihydroxyprogesterone (**17**)	1	6	10	17	16	
	7.19	11α-Hydroxytestosterone (**14**)	1	46	50	20	24	
	6.10	11α,17β-Dihydroxy-5β-androstan-3-one (**18**)	–	5	7	23	23	

[a] Determined by GC analysis.

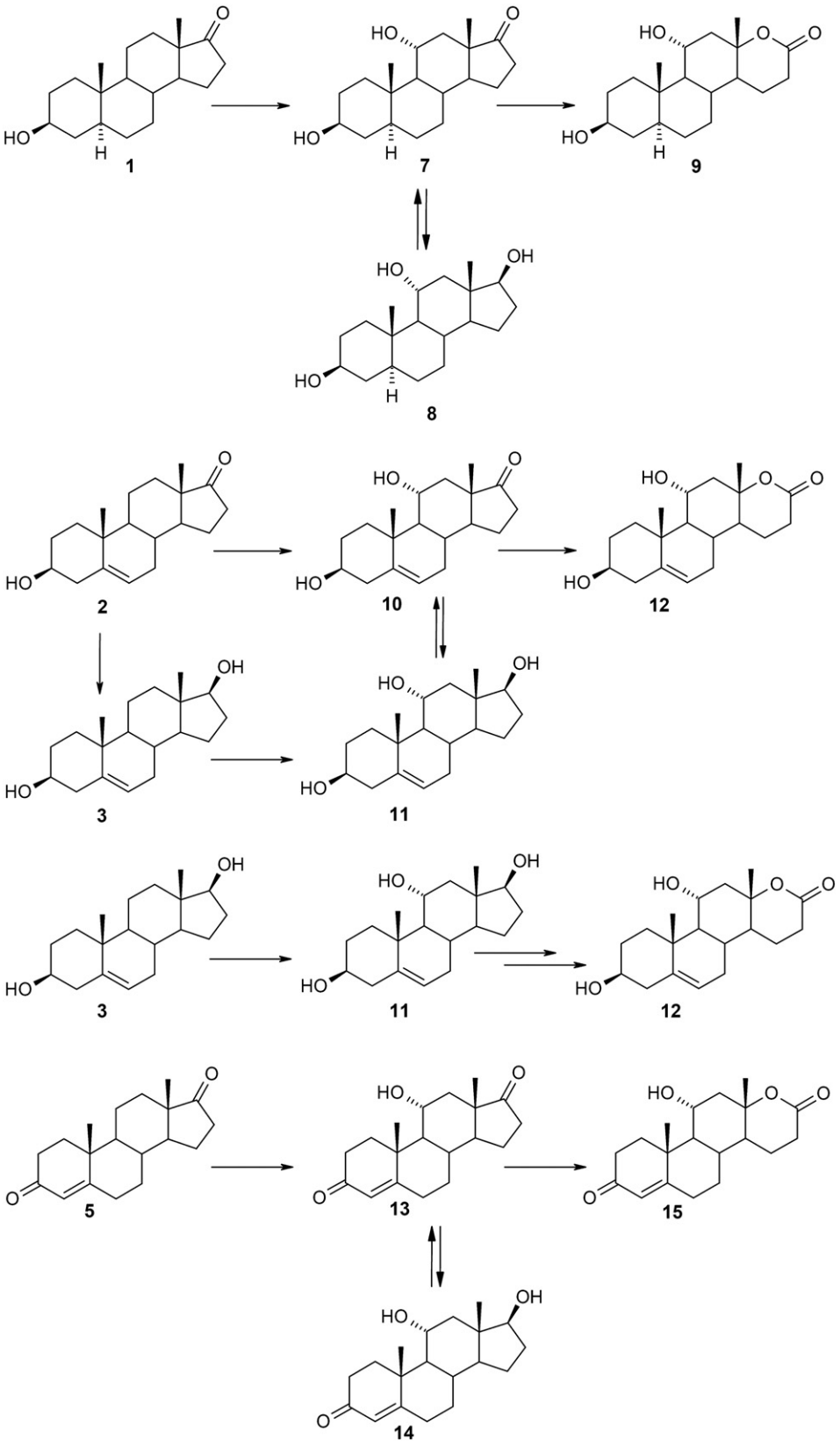

Fig. 3. D-Lactonization pathways of C-19 steroids in *Beauveria bassiana*.

derivatives **7** and **8** grew to 46%, which suggests that the substrate is an inductor of an enzyme catalyzing the 11α-hydroxylation. A similar profile of concentration changes of the 11α-hydroxy derivatives was observed during transformations of the remaining substrates. The amount of 11α-hydroxy derivatives in the reaction mixtures of the tested substrates, sampled at the same time of incubation, was variable and decreased in the following sequence: DHEA (**2**), progesterone (**6**), epiandrosterone (**1**), androstenedione (**5**), androstenediol (**3**).

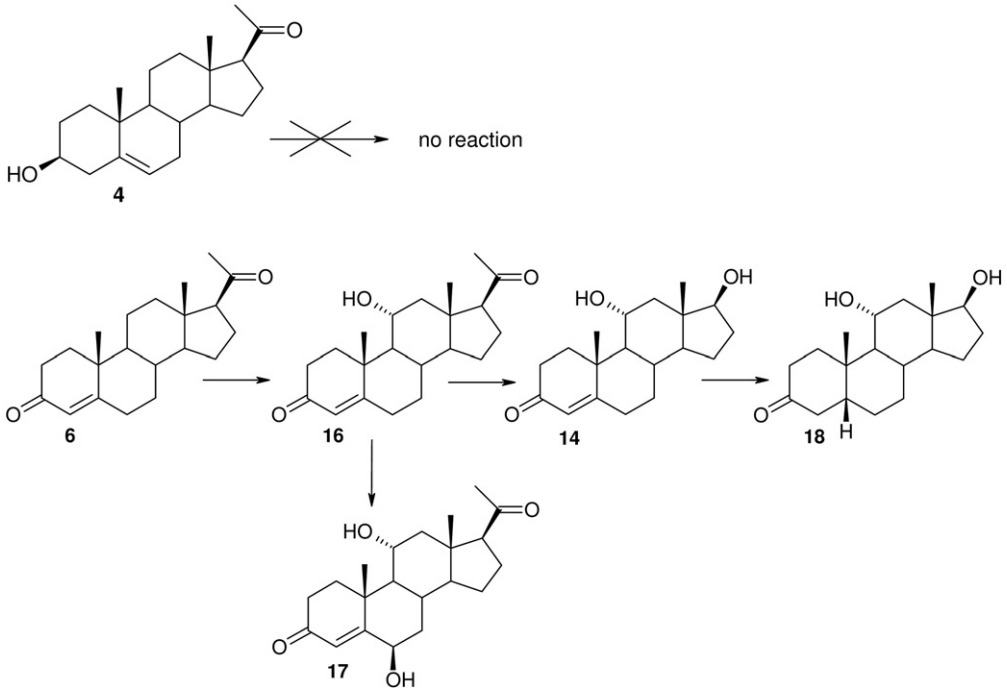

Fig. 4. Metabolic pathways of C-21 steroids in *Beauveria bassiana.*

Comparison of the content of the 11α-hydroxymetabolites in the mixtures indicates that the 17-oxo substrates underwent 11α-hydroxylation noticeably faster. Androstenediol (**3**)—a substrate without carbonyl group—was the slowest to be transformed; after 9 h incubation, only 3% of **3** was hydroxylated, while during the same period, 85% of DHEA was converted into 11α-hydroxy products **10**, **11**, and **12** (Table 4). The inducing abilities of substrates **1** and **3** were confirmed by comparing the transformation efficiency of **3** using either androstenediol (**3**) or equimolar mixture of androstenediol (**3**) and epiandrosterone (**1**) as substrates. After 24 h incubation of mixture of **3** and **1**, the conversion of both substrates was completed, whereas after the same period, only 24% of androstenediol (**3**), when used as a sole substrate, was converted.

In order to confirm the notion that only 11α-hydroxy derivatives undergo the BV oxidation, an experiment was carried out, in which DHEA was added to the *B. bassiana* culture induced by this substrate 9 h earlier (rationale: a culture after the same period of incubation with **2** already exhibited BVMO activity). The reaction mixture after 48 h of transformation contained 65% of 11α-hydroxy-D-lactone **12**, compared to 34% in the standard experiment (Fig. 5). The experiment in which DHEA was introduced into the culture of *B. bassiana* in two equal doses (the second dose was added to the culture exhibiting the hydroxylase and BVMO activity) has shown that the qualitative composition of the metabolites did not change, but the amount of formed hydroxylactone **12** was over two times larger (Table 5).

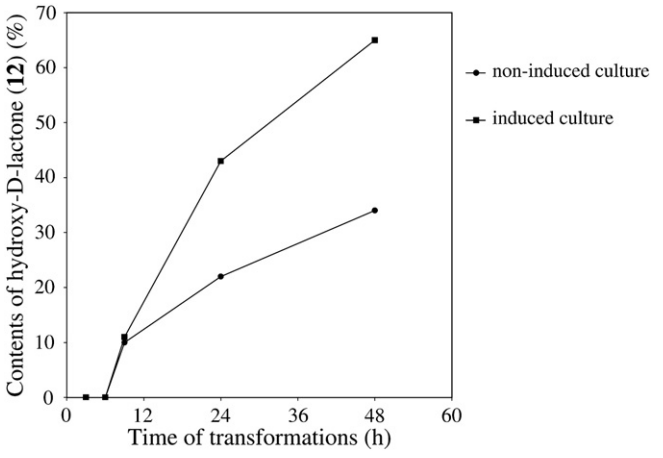

Fig. 5. Comparison of percentage of 3β,11α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (12) in the mixtures after transformation of DHEA by the non-induced and substrate-induced cultures of *Beauveria bassiana*.

4. Discussion

In the culture of the strain *B. bassiana* KCH 1065, the steroidal C-17 ketones were transformed in a one-step process to 11α-hydroxy ring-D δ-lactones. Analysis of composition of the product mixtures as function of incubation time indicates that only the 11α-hydroxy derivatives of the substrates undergo the BV-type oxidation. After 6 h transformation of epiandrosterone (**1**) the reaction mixture contained 11α-hydroxy derivatives **7** and **8** together with 11α-hydroxy-D-lactone **9**, while the 3β-hydroxy-D-lactone—product of the BV

Table 5
Composition of crude mixtures obtained after 24 h of transformation of DHEA by *Beauveria bassiana.*

Compounds (%)[a]	R_t (min)	Transformation by	
		Standard culture	Culture with BVMO activity[b]
DHEA (**2**)	3.46	–	–
Androstenediol (**3**)	3.79	–	–
11α-Hydroxy-DHEA (**10**)	5.11	3	8
3β,11α,17β-Trihydroxyandrost-5-ene (**11**)	5.34	65	45
3β,11α-Dihydroxy-17a-oxa-D-homo-androst-5-en-17-one (**12**)	8.54	22	39
3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one[c]	7.13	nd	nd

[a] Contents of compounds determined by GC analysis.
[b] BVMO activity was confirmed on the basis of presence of lactone **12** in the mixture (detected by GC analysis).
[c] The standard obtained in our previous work [9].

oxidation of epiandrosterone—was not detected, although **1** was the main component of the mixture (Table 4). Mixtures resulting from transformation of DHEA by *B. bassiana* culture with BVMO activity (indicated by the presence of lactone **12** in the mixture) (Table 5) also did not contain detectable amounts of D-lactone without the 11α-hydroxy group, although the DHEA was undoubtedly available to the BVMO. The main component of the mixtures of progesterone metabolites was 11α-hydroxytestosterone (**14**), and testosterone was not identified in any of them, which suggests that also the cleavage of the C-17(20) of this steroid by BV oxidation is preceded by 11α-hydroxylation.

The metabolite mixture resulting from transformation of progesterone did not contain identifiable products of 11α-hydroxytestosterone (**14**) oxidation in the ring D. On the other hand, analysis of composition of the product mixtures after various incubation periods of androstenedione (**5**) has shown that part of the formed 11α-hydroxytestosterone (**14**) was oxidized to the D-lactone **15**; between 24 and 48 h incubation time of **5**, amount of the 11α-hydroxy-D-lactone **15** grew by 12%, and the amount of 11α-hydroxytestosterone decreased by 15% (Table 4). The observed differences of catalytic activity of *B. bassiana* KCH 1065 cultures can stem from various inducing properties of **5** and **6** or their metabolites. Comparison of the metabolism of DHEA (**1**) and androstenediol (**3**) indicates that the 17-oxo substrate is an active inductor of 17β-HSD. During the transformation of progesterone the 17-oxo products are not formed, the culture does not exhibit 17β-HSD activity, the 11α-hydroxytestosterone (**14**) is not oxidized.

It seems that the stage of oxidation of the 17β-alcohol to the ketone is decisive for determining the amount of resulting D-lactone. Until the moment of appearance of the BVMO activity, a major portion of the substrates **1**, **2** and **5** was transformed to the 17β-alcohols **8**, **3** + **11** and **14** respectively. Although DHEA (**2**) was the fastest to be transformed among the studied substrates, the amount of D-lactone in the mixture after 48 h did not exceed 34%, and the main ingredient of the mixture was 3β,11α,17β-triol **11**. During transformation of DHEA by the induced cultures, the amount of 11α-hydroxy-D-lactone **12** reached 65% (Fig. 5). This fact can be explained by assuming that the freshly introduced substrate, after 11α-hydroxylation, is subsequently oxidized to the D-lactone **12** and at the same time, the reduction of ketone at C-17 is limited.

5. Conclusions

The steroidal C-17 ketones were transformed in the culture of the strain *B. bassiana* KCH 1065 in a one-step process to 11α-hydroxy ring-D δ-lactones. Although *B. bassiana* is known to carry out Baeyer–Villiger degradation of ketones (including degradation of 20-keto-pregnanes to 17β-hydroxyandrostanes) [19,22,36,37], up till now, none of the species of the genus *Beauveria* has been applied to steroid lactonization. The BVMO(s) of the strain is distinguished from other enzymes described in the literature, which catalyze BV oxidation of C-20 or C-17 steroid ketones [11,13,16,38,39], by the fact that it oxidizes solely substrates with 11α-hydroxyl group. Steroidal lactones often exhibit useful biological properties, such as anticancer, antiandrogenic, and antihypercholesterolemic activity [40–43]. *B. bassiana* KCH 1065 is a promising fungus that may be used in commercial processes to obtain novel biologically active steroidal hydroxylactones via the biooxidation process.

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