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Baeyer-Villiger oxidation of DHEA, pregnenolone, and androstenedione by *Penicillium lilacinum* AM111

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ABSTRACT

The Baeyer-Villiger monooxygenase (BVMO) produced by *Penicillium lilacinum* AM111, in contrast to other enzymes of this group known in the literature, is able to process 3β-hydroxy-5-ene steroid substrates. Transformation of DHEA and pregnenolone yielded, as a sole or main product, 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one, a new metabolite of these substrates; pregnenolone was transformed also to testololactone. Testololactone was the only product of oxidation of androstenedione by *P. lilacinum* AM111.

Investigations of the time evolution of reaction progress have indicated that the substrates stimulate activity of BVMO(s) of *P. lilacinum* AM111.

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1. Introduction

Recent years have brought, along with intensive research on the role and biological activity of DHEA (3β-hydroxy-androst-5-en-17-one) and pregnenolone, many studies on their metabolism and the role and activity of the metabolites, including those which are not sex hormones [1–11]. DHEA derivatives with oxygen function at C-7, mainly 7α- and 7β-hydroxy-DHEA, were identified in many mammalian organs and tissues (e.g. brain, liver, skin) [1–8,12,13]. 7α-Hydroxy-DHEA was often found as the main metabolite product of microbiological transformations of DHEA [14–22].

In an effort to obtain new metabolites of DHEA and pregnenolone, different from any known derivatives with oxygen function at the C-7, screening tests were carried out and the strain *Penicillium lilacinum* AM111 was selected for further studies.

Literature reports indicate that the strains of genus Penicillium are able to carry out transformations of steroid substrates by means of reduction, hydroxylation or Baeyer-Villiger oxidation [23–32]. *Penicillium decumbens* strain has been used for the reduction of double bonds, particularly the conversion of 4-en-3-oxosteroids to 5α-3-ones [24,25]. In view of the fact that *P. decumbens* is one of only a few microorganisms known to perform this conversion, it occupies a central role in the metabolism of steroids. Some studies focused on 15α-hydroxylation of 3-ethyl-gone-4-en-3,17-dione catalyzed by *Penicillium raistrickii* [26,27]. Various strains of genus *Penicillium* carry out Baeyer-Villiger oxidation [26–32].

Baeyer-Villiger monooxygenases (BVMOs) are flavoenzymes that catalyze the Baeyer-Villiger reaction by insertion of an oxygen atom next to a keto function thus converting ketones to corresponding esters or lactones. BVMOs are produced by numerous bacteria (e.g. of the genera *Actinetobacter*,

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Arthobacter, *Nocardia*, *Rhodococcus*, *Streptomyces*) and fungi (e.g. of the genera *Aspergillus*, *Culvularia*, *Fusarium*, *Penicillium*) [33]. The strain *P. lilacinum* was the source of the first isolated BVMO responsible for oxidation of androstenedione to testololactone [28]. BVMOs carry out Baeyer-Villiger degradation of 17β-acetyl side chain of C-21 substrates and ring D oxidation of androstenedione [33]. Substrates of the reactions described above were usually 4-en-3-oxo steroids.

DHEA and pregnenolone were subjected to transformation by fungus *Penicillium citreo-viride*, which was shown to convert androstenedione and progesterone to testololactone [31]. In a culture of this strain, DHEA was oxidized to testololactone, while pregnenolone was not transformed.

2. Experimental

2.1. Materials

2.1.1. Substrates

DHEA, pregnenolone, androstenedione, and progesterone standard were purchased from Sigma–Aldrich Chemical Co. The microorganism *P. lilacinum* AM111 used in this study was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław. Originally, the AM111 strain was isolated from synthetic fibres.

2.2. Conditions of cultivation and transformation

Fungi were incubated on 3% glucose and 1% aminobac, in 300 ml Erlenmeyer flasks with 100 ml of medium. After cultivation at 25 °C for three days on a rotary shaker, 20 mg of the substrate, dissolved in 1 ml of acetone, was added. Transformation of the substrate was carried out in 5 flasks under the same conditions. The reaction progress was monitored by TLC and GC. Each test was carried out in at least three independent, parallel experiments.

2.3. Isolation and identification of the products

The products of transformation were extracted from the mixtures three times with 20 ml of chloroform. Transformation products were separated by column chromatography on silica gel with ethyl acetate/methylene chloride/acetone (3:1:1) as eluent. TLC was carried out with Merck Kieselgel 60 F_{254} plates using the same eluent. In order to develop the image, the plates were sprayed with solution of methanol in concentrated sulphuric acid (1:1) 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 a HP-1 column cross-linked Methyl Siloxane, 30 m × 0.53 mm × 1.5 μm film thickness. Applied temperature program: 190 °C/1 min, gradient 4 °C/min to 235 °C/5 min and 30 °C/min to 300 °C/3 min; injector and detector temperature was 300 °C. Retention times of the identified compounds are given in Table 1. Infrared spectra were recorded in KBr discs on a Mattson IR 300 Spectrometer. The NMR spectra were measured in $CDCl_3$ and recorded on a DRX 300 MHz Bruker Avance spectrometer with TMS as internal standard. Optical rotation measurements were carried out on Autopol IV automatic polarimeter (Rudolph).

2.4. Biotransformations of DHEA (1) by P. lilacinum AM111

After 36 h incubation of 100 mg DHEA in *P. lilacinum* AM111 culture, 92 mg of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**) have been isolated (Fig. 1).

2.4.1. 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (4)

mp 227–230 °C; $[\alpha]_D^{20} = -93.9$ (c 0.1 in $CHCl_3$); IR ν_{max}(cm^{-1}): 3446, 1716, 1653, 1215; ^{1}H NMR: δ (ppm): 0.96 (s, 19-H_3), 1.30 (s, 18-H_3), 2.59 (m, 16α-H), 2.68 (m, 16β-H), 3.52 (m, W_h = 26.74 Hz, 3α-H), 5.33 (t, 6-H), ^{13}C NMR: δ (ppm): 171.5 (C-17), 140.6 (C-5), 120.8 (C-6), 71.5 (C-3), 83.2 (C-13), 49.0 (C-9), 46.7 (C-14), 41.9 (C-4), 38.9 (C-12), 36.9 (C-1), 36.6 (C-10), 34.4 (C-2), 31.5 (C-7),

Table 1 – Composition of crude mixture obtained in transformation by *P. lilacinum* AM111

Substrate	$R_{t\ (min)}$	Compounds present in mixture (%)[a]	Time of substrate incubation			
			6 (h)	12 (h)	24 (h)	30 (h)
DHEA (**1**)	4.38	DHEA (**1**)	93	66	14	–
	8.04	3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**)	7	34	86	100
Androstenedione (**3**)	5.44	Androstenedione (**3**)	98	85	30	3
	9.40	Testololactone (**5**)	2	15	70	96
Pregnenolone (**2**)	6.43	Pregnenolone (**2**)	85	75	58	51
	7.80	Progesterone (**6**)[b]	15	9	–	–
	4.38	DHEA (**1**)[b]	–	5.5	7	2
	5.44	Androstenedione (**3**)[b]	–	9.5	12	7
	8.04	3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (4)	–	–	15	23
	9.40	Testololactone (**5**)	–	–	3	12

[a] Determined by GC analysis.
[b] Identified in GC and TLC on the basis of standards.

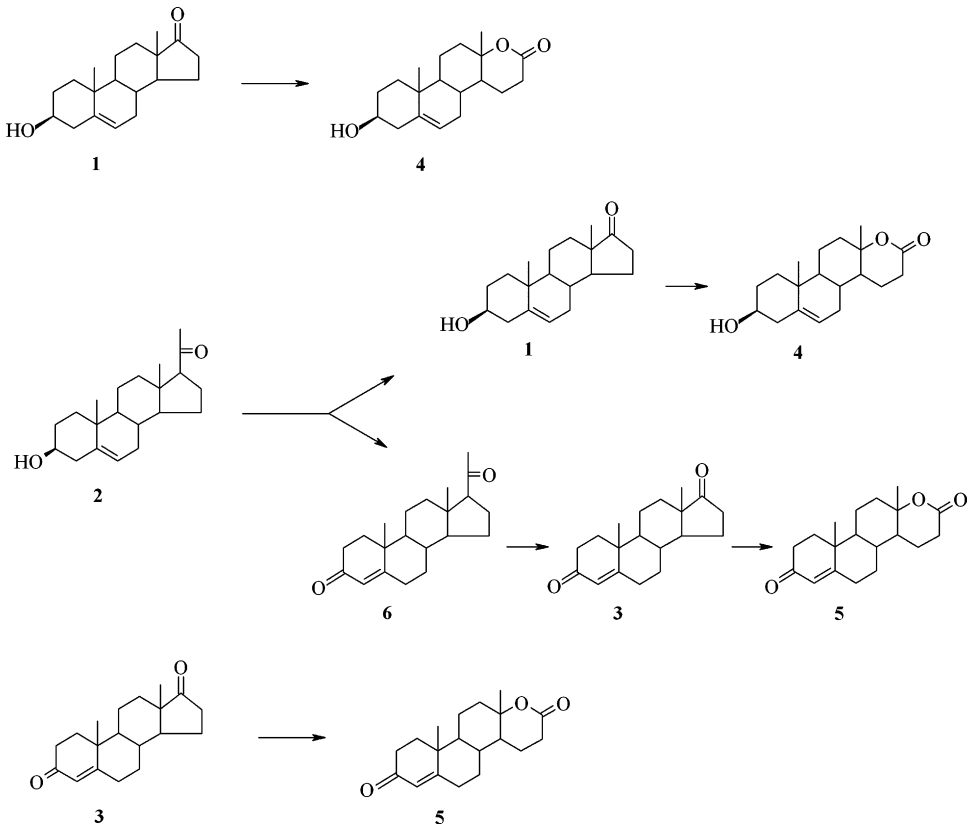

Fig. 1 – Baeyer-Villiger oxidation of DHEA (1), pregnenolone (2) and androstenedione (3); Pathways of pregnenolone transformations: via DHEA or via progesterone to lactones 4 or 5.

31.1 (C-8), 28.8 (C-16), 21.9 (C-11), 20.1 (C-18), 19.9 (C-15), 19.3 (C-19).

2.5. *Biotransformations of pregnenolone (2) by P. lilacinum AM111*

After a three-day transformation of 100 mg of pregnenolone (**2**) the following ingredients were isolated: 28 mg of an unreacted substrate, 42 mg of 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**) and 15 mg of testololactone (**5**) (Fig. 1). No increase in the transformation rate was observed for incubation period longer than three days.

2.5.1. 2.5.1.*Testololactone (5)*

mp 209–210 °C; $[\alpha]_D^{20} = +46.2$ (c 0.16 $CHCl_3$), literature [27] mp 207–209 °C; $[\alpha]_D^{20} = +43$; IR ν_{max} (cm^{-1}): 1716, 1666, 1614, 1214; ^1H NMR δ (ppm): 1.16 (s, 19-H_3), 1.35 (s, 18-H_3), 2.59 (m, 16α-H), 2.68 (m, 16β-H), 5.75 (s, 4-H); ^{13}C NMR: δ (ppm): 199.2 (C-3), 171.1 (C-17), 169.2 (C-5), 124.1 (C-4), 82.7 (C-13), 52.5 (C-9), 45.7 (C-14), 39.1 (C-12), 38.4 (C-10), 38.0 (C-8), 35.5 (C-1), 33.8 (C-2), 32.4 (C-6), 30.5 (C-7), 28.6 (C-16), 21.9 (C-11), 20.1 (C-18), 19.9 (C-15), 17.44 (C-19).

2.6. *Biotransformations of androstenedione (3) by P. lilacinum AM111*

After two-day incubation of 100 mg of androstenedione (**3**) in *P. lilacinum* AM111 culture, 90 mg of testololactone (**5**) were isolated (Fig. 1).

3. Results and discussion

The structures of the obtained products were determined by means of the IR, ^1H NMR and ^{13}C NMR spectra. Assumed structures were confirmed by comparison of the chemical shifts of selected, diagnostic signals in the NMR spectra of the substrate and the products with the literature data. Resonance signals in both ^1H NMR and ^{13}C NMR spectra of **4** were consistent with the formation of the ring D lactone. Downfield shift (35.7 ppm) of the C-13 resonance signal with respect to the substrate is consistent with insertion of an oxygen atom adjacent to this position on the ring D. This is coupled with the downfield shift in the 18-methyl resonance signal of δ_H 0.43 ppm and δ_C 6.6 ppm. Absence of signal of the 17-carbonyl group at δ_C = 221 ppm and presence of a signal at δ_C = 171.5 ppm are consistent with D lactone formation [34]. Proton NMR signals at δ: 3.52(m) and 5.33(t), in analogy to the spectrum of DHEA (**1**), indicate the presence of 3β-OH group and double bond at C-5 in the product. The band at 1716 cm^{-1} in the IR spectrum of the product **4** confirms the δ-lactone structure, and the band at 3446 cm^{-1} indicates that the hydroxyl group is present. The spectral data of **5** were in agreement with those reported in the literature [30,31,35].

The strain *P. lilacinum* AM111 produces the Baeyer-Villiger monooxygenase(s), which is able to carry out ring D oxidation and degradation of 17β-acetyl side chain of 3β-hydroxy-5-ene as well as 4-en-3-oxo steroid substrates. DHEA (**1**) was oxidized to 3β-hydroxy-17a-oxa-D-homo-androst-5-en-

17-one (**4**) as the sole product, while androstenedione (**3**) was converted, with high yield, into testololactone (**5**). Transformation of pregnenolone (**2**), apart from 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**), led to small amounts of testololactone (Fig. 1). Analysis of the reaction mixture composition as function of time indicates that the steroid substrate induces activity of the enzymes responsible for the ring D oxidation as well as for the degradation of 17β-acetyl side chain (Table 1).

After 6 h transformation of DHEA the content of hydroxylactone **4** in the reaction mixture was 7%, and after additional 6 h reached 34%. Androstenedione was converted respectively into 2% and 15% of testololactone.

Among the products of the transformation of pregnenolone (**2**), apart from **4** and **5**, also progesterone (**6**), DHEA (**1**), and androstenedione (**3**) were isolated. The time evolution of the transformation of this substrate indicates that the compound undergoes oxidation reactions of the Baeyer-Villiger type along two pathways: through DHEA (**1**) to hydroxylactone **4** or, after conversion to progesterone (**6**), through androstenedione (**3**) to testololactone (**5**) (Table 1, Fig. 1). Oxidation of **2** and **6** likely proceeds according to the following sequence of reactions: oxidative esterification of 17β-acetyl chain to 17β-acetoxy-3β-hydroxy-androst-5-ene or testosterone acetate, ester bond hydrolysis leading to 17β-alcohols, subsequently oxidized to 17-oxo products (DHEA or androstenedione), which undergo D-ring Baeyer-Villiger oxidation yielding D-ring lactones **4** and **5**. Among the products resulting from the transformation of **2**, only progesterone (**6**), androstenedione (**3**), and DHEA (**1**) were identified in the reaction mixture. In accord with the literature examples of progesterone to testololactone oxidation, testosterone acetate was not identified due to its fast hydrolysis catalyzed by the active esterase present in the culture [36]; testosterone was also usually not identified [31].

The mixture of products after 6 h incubation of pregnenolone (**2**) contained, apart from the substrate, only progesterone (**6**) (ca. 15%) (Table 1). Only after 12 h of the elapsed reaction time, DHEA (**1**) and androstenedione (**3**) were identified – products of the elimination of the 17β-acetyl side chain and subsequent oxidation of the 17β-OH group. Higher content of androstenedione (**3**) in the mixture, as compared to DHEA (**1**), indicates that progesterone (**6**) is oxidized faster than pregnenolone (**2**). 3β-Hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**) is identified in the reaction mixture earlier and in larger amount than testololactone (**5**), which suggests that the BVMO responsible for the ring D oxidation prefers the 3β-hydroxy-5-ene substrate. This assumption is supported by the observed faster oxidation of DHEA (**1**) in comparison with androstenedione (**3**) (Table 1).

The composition of the mixture of pregnenolone (**2**) transformation products indicates that from the moment of identification of Baeyer-Villiger oxidation products in the mixture, the amount of 4-ene-3-oxo products does not increase; their total content, in the investigated incubation period, is close to 15%, i.e. the amount of progesterone in the initial stage of the transformation (Table 1). It is probable that the oxidation of the 17β-OH group, one of the stages in the cycle of reactions leading from pregnenolone to 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**) and progesterone to testololactone (**5**), is competitive with respect to the oxidation of 3β-hydroxyl group, the first stage of the pregnenolone (**2**) transformation to progesterone (**6**).

The compound obtained by us, 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one (**4**), is a new metabolite of DHEA (**1**) and pregnenolone (**2**). The literature examples of microbiological oxidation of Baeyer-Villiger type of both substrates led to testololactone (**5**) [29,31]. One of the strains of the genus *Penicillium* – *P. citreo-viride* oxidized DHEA (**1**) to testololactone (**5**), while it did not exhibit any enzymatic activity towards pregnenolone (**2**) [31]. The strain *P. lilacinum* AM111 used by us synthesizes BVMO(s) which, differently from other enzymes of this group known in the literature, accepts both 3β-hydroxy-5-ene substrates: DHEA (**1**) and pregnenolone (**2**).

Steroidal lactones possess useful biological properties, such as anticancer, antiandrogenic, and antihypercholesterolemic activity [37–40]. The results obtained in this study indicate that the strain of *P. lilacinum* AM111 is a promising fungus that may be used in commercial processes, and which offers a potential new route to novel biologically active steroids.

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