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Biohydroxylation of 7-oxo-DHEA, a natural metabolite of DHEA, resulting in formation of new metabolites of potential pharmaceutical interest

Running title: Biohydroxylation of 7-oxo-DHEA

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ABSTRACT

Metabolism of steroids in healthy and unhealthy human organs is the subject of extensive clinical and biomedical studies. For this kind of investigations, it is essential that the reference samples of derivatives of new derivatives of natural, physiologically active steroids

(especially those difficult to achieve in the chemical synthesis) become available. This paper demonstrated for the first time transformation of 7-oxo-DHEA – a natural metabolite of DHEA, by using *Syncephalastrum racemosum* cells. The single-pulse fermentation of substrate produced two new hydroxy metabolites: 1β,3β-dihydroxy-androst-5-en-7,17-dione, and 3β,12β-dihydroxy-androst-5-en-7,17-dione, along with the earlier reported 3β,9α-dihydroxy-androst-5-en-7,17-dione and 3β,17β-dihydroxy-androst-5-en-7-one. Simultaneously, the same metabolites, together with small quantities of 7α- and 7β-hydroxy-DHEA, as well as the products of their reduction at the C-17 were obtained after transformation of DHEA under pulse-feeding of the substrate. The observed reactions suggested that this microorganism contains enzymes exhibiting similar activity to those present in human cells. Thus, the resulting compounds can be considered as potential components of the eukaryotic, including human, metabolome.

Introduction

For a long time, DHEA (dehydroepiandrosterone) was perceived as an insignificant product of metabolical degradation of steroids, but nowadays it is the object of intensive research. Many of the biological functions of DHEA are associated with the activity of its metabolites, which, like DHEA itself, initially were considered inactive. Among them, 7-oxygenated derivatives are predominantly mentioned - 7α-hydroxy-DHEA and its 7β-hydroxy epimer (1,2). Both compounds, as well as 7-oxo-DHEA – an intermediate in the DHEA→7-hydroxy-DHEA conversion – are identified in many mammalian tissues. 7-Oxo-DHEA was assigned a significant role in the induction and regulation of thermogenesis (3). Also, its beneficial effect in the treatment of obesity is indicated (4). This metabolite is also regarded as a natural antiglucocorticoid (5). In many countries, 7-oxo-DHEA is available commercially under the trade name 7-Keto Fuel® or 7-Keto-DHEA, and is used as a food supplement for athletes to increase the metabolic rate and improve the BMI index (6). There are no unequivocal results indicating that 7-oxo-DHEA can improve mental capacity, although in some studies its precognitive effect was observed, modulating the brain activity and delaying the progression of neurodegenerative diseases (7).

In contrast to DHEA, 7-oxo-DHEA is not a precursor of biologically active androgens or estrogens, which, if present in the body at a level significantly above physiological norm, would increase the risk of hormone-dependent diseases. Thus, this compound is considered to

be a safer steroid that could replace the DHEA therapy (8). As DHEA, it may also have antiproliferative and cytotoxic activity for some tumor cell types (9).

For many years, our team has conducted research on microbial functionalization of compounds of natural origin, including steroid systems (10-20). Among these transformations, the hydroxylation reactions in an inactivated position of a transformed compound are of particular importance. The use of biotransformation as a tool for modification of the molecules makes it possible to obtain derivatives that are found in the nature in minor amounts, as well as new analogues in amounts which not only allow for determination of their structure, but also for biological assays. Biotransformations are often the source of derivatives with enhanced biological activity, and hence with more effective therapeutic effects relative to the parent substrates. Microorganisms express multienzyme systems that ensure regio- and stereoselectivity of the reactions, and products obtained with their participation are frequently not available at the conditions of classical chemical transformations. Biotransformations using microorganisms can be also used as models useful in determining the metabolic pathways of natural and xenobiotic compounds, including drugs, in mammals (21,22).

As it is reported by extensive literature sources, DHEA is transformed in cultures of microorganisms, as well as in mammalian tissues, usually to a mixture of both C-7-epimeric hydroxy derivatives. These microbial transformations have been reviewed recently (23,24). In some of these processes the 7-oxo-DHEA is also identified (16,20,25).

Monitoring of the metabolism of DHEA and its derivatives in mammals would be difficult due to their very low (~ ng/ml) concentration (26), rapid conversion to hormones or inactive conjugates, and a high content of other lipid compounds in the environment. So far, in body fluids of mammals there were identified only a few metabolites of 7-hydroxy-DHEA containing several hydroxyl groups in their structure (27). These compounds showed more than thirty times higher anti-inflammatory activity and higher stability compared to the 7β-hydroxy-DHEA in model studies of immune-dependent diseases such as multiple sclerosis, lung injury, chronic inflammation of the prostate and colon (27). As demonstrated in the study, the main metabolites of 7-oxo-DHEA were the 7-hydroxylated derivatives of DHEA, but at the same time a number of other unidentified structures were also detected (6). The

3β,16α-dihydroxy-androst-5-en-7,17-dione was described as an excretory product in the urine of a patient with adrenal carcinoma (28).

In the light of the reasoning above it is justified to conduct studies on the possibilities of formation of new 7-oxo-DHEA derivatives with potential biological activity as a result of microbial transformations. For this purpose, two studies were carried out. The first one included experiments with 7-oxo-DHEA as the biotransformation substrate, and the second one – with DHEA as the substrate. In the second study, influence of the substrate supply mode to the microorganism culture was also investigated. The earlier literature reports on transformations of natural compounds such as diosgenin (29) and ursolic acid (30,31) encouraged us to the use of filamentous fungus *Syncephalastrum racemosum*. Another indication for this microorganism was that it has minimal human pathogenicity (32), making it a good candidate to be investigated in the bioconversion of products for potential pharmacological use.

Methods and Materials

Chemicals

3β-Hydroxy-androst-5-en-7,17-dione (**1**) was prepared from the DHEA 3-acetate by oxidation with chromium(VI) oxide and subsequent base-catalyzed hydrolysis of 3β-acetoxy-androst-5-en-7,17-dione (7-oxo-DHEA acetate). DHEA 3-acetate, DHEA (**6**) and androstenediol (3β,17β-dihydroxyandrost-5-ene) were purchased from Sigma-Aldrich Chemical Co. Chemical standards: 7α-hydroxy-DHEA (**7**), 7β-hydroxy-DHEA (**8**), 3β,7α,17β-trihydroxy-androst-5-ene (**9**), 3β,7β,17β-trihydroxy-androst-5-ene (**10**) and 3β,17β-dihydroxy-androst-5-en-7-one (**5**) were synthesized in our previous work (16).

Preparation of 3β-hydroxy-androst-5-en-7,17-dione (7-oxo-DHEA) (1)

To a solution of the DHEA 3-acetate (800 mg) in 32 ml of chloroform at 60 °C, a mixture of acetic acid (8 mL), acetic anhydride (2 mL) and CrO_3 (6 g) in chloroform (44 mL) was added dropwise and then the mixture was stirred for further 2 h at 60 °C. After cooling to room temperature, the solution was treated with 140 mL of 10% oxalic acid and extracted with diethyl ether three times. The organic layers were combined, washed with water and saturated $NaHCO_3$. After drying ($MgSO_4$) and evaporation of solvent, crude 7-oxo-DHEA acetate was saponificated with Na_2CO_3 in methanol according to the procedure described earlier (33). The

mixture of products was subjected to column chromatography in hexane:acetone (2:1 v/v) as eluent. 7-Oxo-DHEA (307 mg, 42% yield) was found to be in excess of 99.1 % purity following GC and elemental analysis, anal. C 75.49, H 8.72%, calcd for $C_{19}H_{26}O_3$ C 75.46, H 8.67%.

Microorganism

The fungal strain, *Syncephalastrum racemosum* AM105, used for biotransformation was obtained from the collection of the Department of Pharmaceutical Biology and Botany of the Wrocław Medical University, Poland. It was maintained on Sabouraud 4% dextrose-agar slopes and freshly subcultured before use in the transformation experiments.

Conditions of cultivation and transformation

Bioconversions of substrates were conducted according to the standard two-stage fermentation procedure. Erlenmeyer flasks (300 mL), each containing 100 mL of a medium consisting of glucose 30 g L^{-1} and aminobak 10 g L^{-1}, were inoculated with 5 mL of starter cultures and incubated for 3 days at 25 °C in a rotary shaker. The same conditions were used for the biotransformation stage. After growth period of microorganism, substrate as a 5% solution in acetone was added to each of the cultures (single-feeding), to give a final substrate concentration of up to 0.50 g L^{-1}. The experiment was performed with four replications. Incubation was continued for up to 96 h after addition of the substrate. Alternatively, a 5% solution of the substrate was pulse-fed to the growing fungus in portions of 10, 20, 30 and 40% at 48, 60, 72 and 84 h, respectively, after inoculation. The fermentation was allowed to proceed for 3 days (after last feed).

Isolation and identification of products. At the end of incubation period, the cells were collected by centrifugation and washed with 0.05 M sodium phosphate buffer (pH 7.0). Mycelia and broth were extracted three times with chloroform. The organic extracts were combined, dried over anhydrous magnesium sulfate, filtered, and the solvent was subsequently evaporated *in vacuo* to give a brown gum. This crude extract was analyzed by TLC and GC and then chromatographed on a column of silica with the same eluent as for TLC.

TLC was carried out with Merck Kieselgel 60 F_{254} plates and visualized by spraying them with a mixture of methanol in concentrated sulfuric acid (1:1 v:v) and heating to 120 °C until the colors developed. 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 DB-5MS column, 30 m x 0.320 mm x 0.25 μm. The applied temperature program was 220 °C/1 min, gradient 2 °C/min to 270 °C and then 30 °C/min to 300 °C/5 min; injector and detector temperature was 300 °C. IR spectra were determined using Mattson IR 300 Thermo Nicolet spectrophotometer using KBr pellets. The NMR spectra were measure in $CDCl_3$. The spectra were recorded on a Bruker Avance ^{TM}300 MHz spectrometer. 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. Elemental analysis was performed on Vario EL III analyzer.

Products isolated in the course of transformation of 7-oxo-DHEA (1)

After 96 h transformation of 7-oxo-DHEA (**1**) (0.2 g), yielded mycelial and broth extracts were combined and purified on silica gel with the use of a mixture of acetone:ethyl acetate:methylene chloride:2-propanol (0.3:1.5:1.5:0.2 v/v/v/v) as eluent. The following compounds were isolated (% mol): 1β,3β-dihydroxy-androst-5-en-7,17-dione (**2**) (34 mg, 18%), 3β,12β-dihydroxy-androst-5-en-7,17-dione (**3**) (20 mg, 11%), 3β,9α,-dihydroxy-androst-5-en-7,17-dione (**3**) (40 mg, 21%) and 3β,17β-dihydroxy-androst-5-en-7-one (**5**) (12 mg, 6%).

1β,3β-Dihydroxy-androst-5-en-7,17-dione (2)*:* amorphous solid; $C_{19}H_{26}O_4$: calcd. C,71.67; H, 8.23; found. C, 71.91; H, 8.39%; IR (KBr) ν_{max} 3520, 1740, 1673, 1630, 1408 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.90 (3H, s, H-18), 1.25 (3H, s, H-19), 3.61 (1H, dd, *J* = 11.4 Hz, 4.4 Hz, H-1α), 3.70-3.76 (1H, m, H-3α), 5.85 (1H, br s, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.5 (C, C-17), 200.9 (C, C-7), 162.8 (CH, C-5), 128.1 (CH, C-6), 75.9 (CH, C-1), 66.3 (CH, C-3), 50.2 (CH, C-9), 47.4 (C, C-13), 45.5 (CH, C-8), 44.8 (CH, C-14), 44.5 (C, C-10), 42.2 (CH_2, C-2), 41.7 (CH_2, C-4); 35.5 (CH_2, C-16); 30.9 (CH_2, C-12), 24.2 (CH_2, C-15), 23.1 (CH_2, C-11), 13.8 (CH_3, C-18), 11.7 (CH_3, C-19).

3β,12β-Dihydroxy-androst-5-en-7,17-dione (3)*:* amorphous solid; $C_{19}H_{26}O_4$: calcd. C,71.67; H, 8.23; found. C, 71.70; H, 8.43%; IR (KBr) ν_{max} 3570, 1742, 1670, 1633, 1400 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.98 (3H, s, H-18), 1.24 (3H, s, H-19), 3.66-3.71 (1H, m, H-3α), 3.78 (1H, dd, *J* = 11.2 Hz, 4.7 Hz, H-12α), 5.75 (1H, br s, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 222.2 (C, C-17), 200.4 (C, C-7), 166.1 (CH, C-5), 125.9 (CH, C-6), 72.1 (CH, C-3), 70.3 (CH, C-12), 51.4 (C, C-13), 48.7 (CH, C-9), 43.9 (CH, C-8), 43.4 (CH, C-14), 41.8 (CH_2, C-

4), 38.5 (C, C-10), 36.3 (CH_2, C-1), 35.5 (CH_2, C-16); 31.1 (CH_2, C-2), 28.1 (CH_2, C-11), 24.0 (CH_2, C-15), 17.3 (CH_3, C-19), 8.1 (CH_3, C-18).

3β,9α-Dihydroxy-androst-5-en-7,17-dione (4)*:* amorphous solid; $C_{19}H_{26}O_4$: calcd. C, 71.67; H, 8.23; found. C, 71.72; H, 8.31%, IR (KBr) ν_{max} 3452, 1740, 1670, 1629, 1406 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.90 (3H, s, H-18), 1.38 (3H, s, H-19), 3.68-3.74 (1H, m, H-3α), 5.82 (1H, br s, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 220.3 (C, C-17), 199.0 (C, C-7), 166.7 (CH, C-5), 125.8 (CH, C-6), 78.4 (C, C-9), 69.6 (CH, C-3), 49.3 (CH, C-8), 47.4 (CH, C-13), 44.2 (C, C-10), 41.8 (CH_2, C-4), 40.6 (CH, C-14), 35.6 (CH_2, C-16), 30.8 (CH_2, C-2), 28.7 (CH_2, C-1); 26.6 (CH_2, C-11), 26.6 (CH_2, C-12), 23.7 (CH_2, C-15), 20.7 (CH_3, C-19), 12.9 (CH_3, C-18).

3β,17β-Dihydroxy-androst-5-en-7-one (5): white plates; $C_{19}H_{28}O_3$: calcd. C,74.96; H, 9.27; found. C, 74.56; H, 9.36%; IR (KBr) ν_{max} 3310, 1660, 1630, cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 0.75 (3H, s, H-18), 1.20 (3H, s, H-19), 3.63-3.70 (1H, m, H-3α), 3.65 (1H, t, J=10.7 Hz, 17α-H),.5.67 (1H, br s, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 200.9 (C, C-7), 165.7 (C, C-5), 126.0 (CH, C-6), 81.0 (CH, C-17), 70.5 (CH, C-3), 49.9 (CH, C-9), 45.3 (CH, C-8), 45.1 (CH, C-14), 45.0 (CH, C-14), 43.5 (C, C-13), 41.8 (CH_2, C-4), 38.4 (C, C-10), 36.2 (CH_2, C-1), 35.5 (CH_2, C-12); 31.2 (CH_2, C-2); 30.9 (CH_2, C-16), 25.7 (CH_2, C-15), 21.0 (CH_2, C-11), 17.4 (CH_3, C-19), 11.0 (CH_3, C-18).

Products isolated in the course of transformation of DHEA (6)

After 48 h transformation of DHEA (0.2 g), the resulting mycelial and broth extracts were combined and purified on silica gel with the use of a mixture of acetone:ethyl acetate:methylene chloride (0.5:1.5:1 v/v/v) as eluent. The following compounds were isolated (% mol): 7α-hydroxy-DHEA (**7**) (22 mg, 10%), 7β-hydroxy-DHEA (**8**) (32 mg, 15%), 3β,7α,17β-trihydroxy-androst-5-ene (**9**) (12 mg, 5%), 3β,7β,17β-trihydroxy-androst-5-ene (**10**) (14 mg, 6%). The NMR data of these compound were agreed with the literature (16) and their R_t and R_f were identical with standards available in our laboratory.

Time course experiments. The progress of the reaction was monitored by GC analysis. At regular intervals, 5-mL samples of the conversion medium were taken, extracted and analyzed by comparison of the GC and TLC data with those of authentic samples. Conditions of the reaction were identical to those in the main biotransformation experiments.

Results and Discussion

7-Oxo-DHEA (**1**) was prepared from DHEA 3-acetate by oxidation with chromium(VI) oxide and subsequent base-catalyzed hydrolysis of 3β-acetoxy-androst-5-en-7,17-dione (7-oxo-DHEA acetate). The identity of 7-oxo-DHEA was confirmed by comparison of its spectroscopic data with those of an authentic standard synthesized by us in our previous studies (16).

With the synthesis of the substrate completed, our attention was turned toward its biotransformation by *S. racemosum*. Three-days incubation of **1** with this microorganism yielded several potential metabolites based on GC analysis comparison with the control extracts, but only four metabolites were present in sufficient quantity for characterization (Figure 1).

The structures of these compounds were determined as a result of comparison of their spectroscopic data with that of the starting material (**1**). Thus, the ^{1}H NMR spectrum of metabolite **2** exhibited a new resonance at δ_H 3.60 ppm (dd, J = 4.4 Hz, J = 11,4 Hz). The shape of this signal suggested that it belongs to an axial proton coupled with only two protons, therefore it can be either the signal of 1α or 12α proton. The presence of multiplet at δ_H 3.69-3.75 ppm (m) and olefinic resonance at δ_H 5.84 ppm confirmed that the molecule retains the 3β-hydroxy-androstene skeleton. Correspondingly, the ^{13}C NMR spectrum of **2** showed two oxygenated carbon signals at δ_C 75.9 ppm and δ_C 66.3 ppm. The first one showed long-range (HMBC) correlations with H-19 (s, δ_H 1.24 ppm), and should be assigned to C-1. Additionally, in the HMBC spectrum, the proton signal at δ_H 1.24 ppm correlated with the carbons at δ_C 50.2 ppm (C-9) and δ_C 162.8 ppm (C-5). The NOE enhancement of H-1 (δ_H 3.60 ppm) with H-3 (δ_H 3.72 ppm) was observed in the NOESY spectrum indicating that the 1-OH should be located in the β-configuration. Further evidence for 1β-hydroxylation was provided by downfield shifts of C-2 (Δ 11.1 ppm) and C-10 (Δ 8.2 ppm) signals. This was coupled with upfield shift of C-19 signal from δ_C 17.4 ppm to δ_C 11.7 ppm due to a *γ-gauche*

effect. All these results fully confirmed the structure of metabolite **2** as 1β,3β-dihydroxy-androst-5-en-7,17-dione. The ^{1}H NMR spectrum of **3** revealed a new signal at δ_H 3.78 ppm indicating hydroxylation. The characteristic multiplicity of this signal (dd) suggested the hydroxyl group could be introduced at 12-position. It was confirmed by downfield shift for C-11 (Δ 7.6 ppm) and C-13 (Δ 3.6 ppm). The β (equatorial) stereochemistry of the OH group at C-12 was deduced from the γ-upfield shift of Me-18 from δ_C 13.7 ppm to δ_C 8.1 ppm and ^{3}J interaction between the proton signals of C-18 methyl group resonating at δ_H 0.98 ppm and C-12 at δ_C 70.3 ppm. The rest of the proton and carbon signals was quite similar to the substrate **1** with upfield shift (Δ 0.08 ppm) for the 18-methyl protons' signal. Thus, the structure of metabolite **3** was deduced to be 3β,12β-dihydroxy-androst-5-en-7,17-dione. The new chemical shift at δ_C 78.4 ppm in ^{13}C NMR of **4** suggested the presence of a hydroxyl group, whilst no proton resonance was observed in ^{1}H NMR spectrum in the range of 3.5-4.5 ppm. The data obtained confirmed that the hydroxyl group should be attached to a tertiary carbon. The downfield shifts of signals: C-10 (Δ 5.8 ppm), C-11 (Δ 6.0 ppm), C-8 (Δ 3.6 ppm), and *γ-gauche* upfield shifts of C-1 (Δ 7.6 ppm), C-12 (Δ 3.5 ppm), C-14 (Δ 3.7 ppm) signals of this metabolite in comparison to the spectrum of substrate **1**, indicated hydroxylation at the 9α position. The proposed structure, 3β,9α-dihydroxy-androst-5-en-7,17-dione (**4**), was supported by the downfield shift of the C-19 methyl signal from δ_H 1.23 ppm to δ_H 1.38 ppm, with respect to **1**, that was consistent with the reference shift value (34) The spectroscopic data of **4** were in agreement with those in the literature (35). Structure of the product **5** was determined on the basis of its ^{1}H NMR shift values being identical to the literature data of the standard (16).

Composition of crude mixture obtained after transformation of 7-oxo-DHEA is presented in Table 1. As is apparent from these data, 7-oxo-DHEA underwent mainly hydroxylations; no reduction of the conjugated 7-ketone was observed. All of the biochemical attack processes observed in the transformation of this substrate occurred with equatorial stereochemistry (1β-,12β-) with exception of axial functionalization of tertiary carbon at the 9α-position. Aside from oxidation, a minor reduction pathway was observed with transformation of the C-17 ketone to a C-17β-alcohol generating 3β,17β-dihydroxy-androst-5-en-7-one (**5**). Two of the three hydroxy derivatives of 7-oxo-DHEA are new compounds (**2** and **3**). 1β-Hydroxylase activity, although rarely demonstrated by microorganisms, has been observed in the transformation of the ursolic acid by *S. racemosum* (31). The metabolites containing a hydroxyl group at 9α or 12β position were isolated after transformation by this

microorganism of some less common steroids of the androstane series (35) and diosgenin (29). 3β,9α-Dihydroxy-androst-5-ene-7,17-dione (**4**) with a yield of 37% was previously obtained as a sole transformation product of 7-oxo-DHEA 3β-acetate by *Absidia regnieri* (35).

In order to determine if the obtained metabolites of 7-oxo-DHEA could be final metabolites of DHEA, we used DHEA (**6**) as a test substrate. It was found that cultures of *S. racemosum* under the same conditions metabolize **6** to known compounds identified as 7α-hydroxy-DHEA (**7**), 7β-hydroxy-DHEA (**8**), 3β,7α,17β-trihydroxy-androst-5-ene (**9**) and 3β,7β,17β-trihydroxy-androst-5-ene (**10**) by comparison of their spectroscopic data with the literature values (16) and on the basis of identity of their R_t from GC and R_f from TLC with standards available in our laboratory. Based on the time course experiment analysis, it was found that both allylic alcohols derived from DHEA appeared at the same time and their contents in the reaction mixture rose rapidly during the first hours of the transformation (Table 2). After two days, also reduction products of the carbonyl group at C-17 of these hydroxy derivatives were present in the extracts as well as small quantities of 7-oxo-DHEA (**1**).

Androstenediol (3β,17β-dihydroxy-androst-5-ene) was not found in any of the mixtures, which may suggest that the identified triols (**9** and **10**) arise directly from the 7α-hydroxy- (**7**) and 7β-hydroxy-DHEA (**8**). More than five times lower content of 7-oxo-DHEA (**1**) indicates that further metabolism of the resulting allyl alcohols is mainly connected with the activity of 17β-HSD. The consequence of this is also a small content of hydroxy derivatives of 7-oxo-DHEA (**2**-**4**) in the extracts which, after four-days transformation, reached a total max. value of 12%.

The obtained results prompted us to carry out an experiment in which the method of administration of DHEA was modified (single- vs. pulse-feeding method), having in mind the fact that most microbial hydroxylases are enzymes induced by the presence of the substrates specific to these enzymes (36-38). On the other hand, the feeding-pulse protocol could extend the viability of the cells and their suitability for biotransformation processes (39). In the modified regime of DHEA supply to the microbial cells, the same metabolites were obtained as in the standard transformation, however, the quantitative composition of the mixtures of metabolites in both experiments differed significantly (Table 2). Under the substrate pulse-

feeding conditions, the content of the hydroxy derivatives containing 7-oxo group was more than three times higher. Moreover, the profile of these metabolites was the same as that obtained from 7-oxo-DHEA as the starting transformation substrate.

Conclusion

In summary, the undertaken biotransformations of 7-oxo-DHEA resulted in formation of two new hydroxy derivatives: 1β,3β-dihydroxy-androst-5-ene-7,17-dione (**2**) and 3β,12β-dihydroxy-androst-5-en-7,17-dione (**3**), as well as two known compounds: 3β,9α-dihydroxy-androst-5-ene-7,17-dione (**4**) and 3β,17β-dihydroxy-androst-5-en-7-one (**5**). To the best of our knowledge, this is the first report on biotransformation of 7-oxo-DHEA. The studies also demonstrated that the hydroxy derivatives of 7-oxo-DHEA may be simultaneously the final metabolites of DHEA transformation. Reactions observed during the transformation of DHEA suggest that the studied microorganism contains enzymes exhibiting similar activity to those present in animals and human eukaryotic cells. We are convinced that the achieved high conversion rate of this substrate combined with minimal human pathogenicity of *S. racemosum*, after process optimization, can lead to new approaches for the efficient biosynthesis of steroids with potential biological activity.

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Conflict of Interest

All authors declare no conflict of interest.

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Figure legends

Figure 1. Hydroxylation of 7-oxo-DHEA (**1**) by *S. racemosum*.

Table 1. Metabolites identified following transformation of 7-oxo-DHEA

Compounds	R_t (min)	%[a]
7-oxo-DHEA (**1**)	9.83	7
1β,3β-dihydroxy-androst-5-en-7,17-dione (**2**)	15.31	24
3β,12β-dihydroxy-androst-5-en-7,17-dione (**3**)	11.77	17
3β,9α-dihydroxy-androst-5-en-7,17-dione (**4**)	13.29	32
3β,17β-dihydroxy-androst-5-en-7-one (**5**)	10.25	8

[a]contents of compounds in crude extracts determined by GC analysis.

Table 2. Products accumulation during the conversion of DHEA by *S. racemosum* depending on the time of transformation and the method of the substrate feeding.

Time (h)	Steroids, contents (%)[a]										
	DHEA (**6**)	Hydroxyderivatives[b]				7-oxo-DHEA (**1**)	**2**	**3**	**4**	**5**
		7	**8**	**9**	**10**					
6[c]	84	4	9	-	-	-	-	-	-	-
12[c]	24	38	36	-	-	-	-	-	-	-
48[c]	-	26	34	11	16	4	-	1	2	-
96[c]	-	21	29	10	13	6	2	4	6	2
96[d]	-	3	5	8	7	8	10	15	27	7

[a]determined by GC analysis in crude extracts. [b]standards obtained in our previous work (16). [c]single-feeding of substrate. [d]pulse-feeding of substrate