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Formation of Hydroxylated Steroid Lactones from Dehydroepiandrosterone by *Spicaria fumoso-rosea* F-881

T. G. Lobastova, S. M. Khomutov, and M. V. Donova

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia

e-mail: lobastova@ibpm.pushchino.ru

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Abstract—The transformation of dehydroepiandrosterone by *Spicaria fumoso-rosea* VKM F-881 produced 7α- and 7β-hydroxy-dehydroepiandrosterone, 3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one, and 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one. The yield of the main product—3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one—was 49.5–72 mol % at substrate loadings of 5–20 g/L. Lactone formation proceeded through 7α- and 7β-hydroxy derivatives of dehydroepiandrosterone. The structure of the products was determined by mass spectrometry, ^{1}H-NMR spectroscopy, and ^{13}C-NMR spectroscopy. The proposed microbiological method for producing steroid lactones opens prospects for the synthesis of novel steroid compounds.

Keywords: *Spicaria*, steroids, dehydroepiandrosterone, 3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one, 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one

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During modification of the steroid structures using microorganisms, the Baeyer–Villiger reaction is of particular interest. This reaction introduces the ketone oxygen atom between two carbon atoms, one of which is carbonyl. As a rule, a reaction of this type is carried out by filamentous fungi during degradation of the steroid structure. It is initiated at the D ring and leads to the formation of D-homo lactones, e.g. testololactone. The initial data on the formation of testololactone from progesterone by microorganisms appeared in 1953 [1, 2]. Since then, intensive research of Baeyer–Villiger steroid oxidation by microorganisms has continued. The ability to oxidize ketosteroids to form 3-oxo-steroid-D-homo-lactones was detected in filamentous fungi of different taxonomic positions: *Aspergillus* [3–5], *Penicillium* [6–9], *Trichoderma* [10], and *Fusarium* [11, 12].

The enzymes performing the reactions that introduce an oxygen atom in the structure of ketones belong to the group of so-called Baeyer–Villiger monooxygenases (BVMO). Although BVMO activity is found in many microorganisms, only several BVMOs from bacteria and lower eukaryotes have been characterized to date [13, 14].

Typically, the BVMO substrates are 3-oxo-4-en-steroids; however, the data on the oxidation of 3 (α/β)-hydroxy-steroids with the formation of 3(α/β)-hydroxy-17a-oxa-D-homo-lactones also exist [15, 16]. The formation of lactones from C_{19}- and C_{21}-steroids with a 3β-hydroxy-5-ene structure that proceeds without A ring modification was demonstrated for *Penicillium lilacinum* AM 111 [8] and *P. camemberti* AM 83 [17]. These strains are able to convert pregnenolone and 3β-hydroxy-5-androsten-17-one (DHEA) to 3β-hydroxy-17a-oxa-D-homo-androst-5-en-17-one. The initial stage of pregnenolone bioconversion comprised the degradation of the pregnane side-chain with the formation of DHEA as an intermediate; the stage was followed by the DHEA oxidation to 3β-hydroxylactone. It should be noted that, among the products of the pregnenolone transformation by these strains, not only 3β-hydroxy derivatives but also compounds with a 3-keto-4-ene structure were found: progesterone, androstenedione, and testololactone. The possibility of different pathways for pregnenolone conversion—both through DHEA and through progesterone—was demonstrated [8, 17]. It was found that the DHEA transformation by a *Beauveria bassiana* strain initially proceeded through 11α-hydroxylation of DHEA with the formation of a hydroxy compound that was subjected to Baeyer–Villiger oxidation at a D ring [18]. D-ring lactonization in DHEA by a *Aspergillus tamari* KITA culture was accompanied by 7α-hydroxylation [19].

The presence of a functional group at C-11 of steroids contribute to their D-lactonization by the strain *A. tamari* KITA (QM 1223); D-lactonization is not observed when the substitutions are at other positions.

For example, when the keto group is at the C-6 position, the preferential process is a reduction of the 4,5-double bond in the A ring [5]. The production of 11α-OH-testololactone from 11α-hydroxyprogesterone [5, 6] and the production of 11β-hydroxy-19-nor-testololactone from 11β-hydroxy-19-nor-testosterone [20] by filamentous fungi has been described. The BVMO from the *B. bassiana* strain only oxidized 11α-hydroxylated C-20 and C-17-ketones [18].

Steroidal lactones—the products of Baeyer–Villiger reactions—have anticancer, antibacterial, and antiandrogenic activities [21–24]. The study of the testololactone biological activity demonstrated that the steroid belongs to the group of human aromatase inhibitors and potentially can be used for the treatment of estrogen-dependent breast cancer [23]. Additionally, aromatase inhibitors with a lactone structure are used to study the role of estrogen in age-related changes in humans [25, 26].

An important task is to develop new synthetic pathways to produce structural analogs of testololactone. Filamentous fungi able to convert DHEA have been screened previously; as a result, a strain of *Spicaria fumoso-rosea* VKM F-881 was found. The strain effectively converts DHEA with the formation of a number of steroid products, presumably steroidal lactones [27].

The goal of the work is to study the DHEA transformation performed by the strain of *S. fumoso-rosea* strain VKM F-881 and to evaluate the performance of microbiological preparation of steroid lactones with a 3β,7(α/β)-diol-5-ene structure at high substrate loading.

METHODS

Materials. We used DHEA (Sigma, USA) and 7α-hydroxy dehydroepiandrosterone (3β, 7α-dihydroxy-5-androsten-17-one, **7α-OH-DHEA**) (Schering AG, Germany) with a purity of not less than 95%; we used corn (Sigma-Aldrich, USA) and soybean (cultimed) (Panreac, Spain) extracts. 7β-Hydroxy dehydroepiandrosterone (3β,7β-dihydroxy-5-androsten-17-one, **7β-OH-DHEA**) was prepared as described previously [27]. Other materials and solvents were of reagent grade and analytical grade (Russia).

Microorganisms and their culturing. A strain of *S. fumoso-rosea* VKM F-881 was obtained from the All-Russia Collection of Microorganisms (VKM IBPM RAS). The strain was maintained on wort agar. Cultivation was performed at 29–30°C on a shaker (200-220 rpm) in Erlenmeyer flasks (750 mL) with 50 mL potato-glucose medium (medium **1**) of the following composition (g/L): glucose—20; K_2HPO_4—15.2; KH_2PO_4—18.1; and potato extract up to one liter (pH 6.5).

Transformation of DHEA, 7α-OH-DHEA, and 7β-OH-DHEA. After 48 h of cultivation, 5 mL of culture was transferred to 50 mL medium **2** of the following composition (g/L or mL/L): sucrose—50; KH_2PO_4—5; corn steep liquor (liquid)—20; distilled water—up to one liter (pH 6.5). DHEA substrate (2.0 g/L) in the form of a crystalline powder was added to the medium prior to sterilization. The substrates 7α- and 7β-OH-DHEA (1.0 g/L) in ethanol were added simultaneously with the inoculum. The ethanol concentration in the medium did not exceed 1.0% (vol). The transformation was performed in Erlenmeyer flasks (750 mL) at 29-30°C using a rotary shaker (200–220 rpm).

Cultivation of the strain and DHEA transformation at high concentrations (5–20 g/L). The culture was grown for 48 h (the first stage of culturing) in Erlenmeyer flasks (750 mL) with a medium **3** (50 mL) of the following composition (g/L): sucrose—20; soy flour—10; soy peptone—10; corn steep liquor—5; KH_2PO_4—4; $MgSO_4$—1; and $FeSO_4$—0.01 (pH 6.8–7.0). The resulting seed material (5 mL) was inoculated into an Erlenmeyer flask (750 mL) with the medium **3** (50 mL) and grown for 24 h (the second stage of culturing). The seed material (5 mL) obtained after the second stage of culturing was inoculated into a medium **4** (transformation medium, 50 mL) of the following composition (g/L): sucrose—50; soy peptone—10; KH_2PO_4—5; and $MgSO_4$—5 (pH 6.5). After 24 h of culture incubation in the medium **4**, a DHEA (5–20 g/L) dimethylformamide solution (DMF) containing Tween-80 (0.1%) was added. The concentration of DMF in the medium was 1–3 vol %. The transformation and culturing were carried out at 29–30°C using a rotary shaker (200–220 rpm).

Analysis of steroids. After every 24 h of culturing, one-milliliter samples were taken from the culture liquid. Steroids were extracted with ethyl acetate (5 mL) or, at high substrate loading (DHEA, 5–20 g/L), with ethanol (4–19 mL).

TLC was performed on Sorbofil plates (UF 254, Russia) in the following systems: **A**—benzene–acetone (5 : 3, vol/vol); **B**—benzene–ethyl acetate–acetone (1 : 1 : 1, vol/vol/vol); or **C**—benzene–acetone (3 : 1, vol/vol). Visualization of 3β-hydroxy-5-ene steroids was performed by treating the plates with 4% phosphomolybdic acid in ethanol followed by heating for 3–5 minutes at 60–65°C.

For gas-liquid chromatography (GLC), steroids from the culture liquid (1 mL) were extracted twice with ethyl acetate and evaporated to dryness at 40°C. The residue was dissolved in 0.4 mL of a silanization mixture (hexamethylsilazane–trimethylchlorosilane–pyridine, 4 : 1 : 9) and heated for 40 minutes at 70°C. The analysis was performed on a Hewlett Packard 5890 chromatograph (USA) with a quartz column (15 m × 0.25 mm × 0.2 μm) with a polydimethylsiloxane stationary phase SPB-1 (the carrier gas was helium; the flow rate was 1.4 mL/min, and the column temperature was 150–290°C). A flame ionization detector equipped with a Hewlett–Packard HP 3396A integrator (Hewlett–Packard, USA) was used.

Table 1. Chromatographic and mass-spectrometric characteristics for the major products of DHEA transformation by a strain of *S. fumoso-rosea* VKM F-881

Compound	TLC, R_f			GLC, R_t	Major fragments, *m/z*, (%)	Transformation products
	A	B	C			
DHEA (standard)	0.90	0.91	0.85	11.62	288[M]$^+$(100), 270[M-H_2O]$^+$(34), 255[M-H_2O-CH_3]$^+$(42), 213[M-H_2O-C_3H_5O]$^+$(14), 203[M-C_5H_9O]$^+$(29).	–
7α-OH-DHEA (standard)	0.42	0.64	0.26	12.17	304[M]$^+$(11), 286[M-H_2O]$^+$(100), 271[M-H_2O-CH_3]$^+$(10), 253[M-2H_2O-CH_3]$^+$(6), 243[M-H_2O-CH_3-CO]$^+$(1), 229[M-H_2O-CH_3-$COCH_2$]$^+$(3).	–
1	0.42	0.64	0.26	12.17	304[M]$^+$(9), 286[M-H_2O]$^+$(100), 271[M-H_2O-CH_3]$^+$(10), 253[M-2H_2O-CH_3]$^+$(4), 243[M-H_2O-CH_3-CO]$^+$(1), 229[M-H_2O-CH_3-$COCH_2$]$^+$(2).	7α-OH-DHEA
2	0.51	0.75	0.35	12.79	304[M]$^+$(11), 286[M-H_2O]$^+$(100), 271[M-H_2O-CH_3]$^+$(11), 253[M-2H_2O-CH_3]$^+$(4), 243[M-H_2O-CH_3-CO]$^+$(1), 229[M-H_2O-CH_3-$COCH_2$]$^+$(2).	7β-OH-DHEA
3	0.37	0.58	0.22	14.81	320[M]$^+$(6), 302[M-H_2O]$^+$(100), 287[M-H_2O-CH_3]$^+$(6), 284[M-2H_2O]$^+$(2), 269[M-2H_2O-CH_3]$^+$(2), 259[M-H_2O-CH_3-CO]$^+$(1), 245[M-H_2O-CH_3-$COCH_2$]$^+$(1).	3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one
4	0.26	0.44	0.11	14.31	320[M]$^+$(5), 302[M-H_2O]$^+$(100), 287[M-H_2O-CH_3]$^+$(7), 284[M-2H_2O]$^+$(<1), 269[M-2H_2O-CH_3]$^+$(3), 259[M-H_2O-CH_3-CO]$^+$(1), 245[M-H_2O-CH_3-$COCH_2$]$^+$(1).	3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one

Mass spectra of the transformation products were obtained using a mass spectrometer Finnigan MATSSQ 710 (USA) with a direct sample injection into an ionization chamber at an ionization energy of 70 eV. ^{1}H-NMR spectra (400 MNz) and ^{13}C-NMR spectra (100.6 MNz) were recorded using a Varian UNITY+400 (USA).

Prior to the study, the steroids were dissolved in $CDCl_3$ or in a mixture of $CDCl_3$ and CD_3OD (^{13}C-NMR spectra for compounds 3 and 4). The signals of the solvents $CHCl_3$ in $CDCl_3$ (^{1}H-NMR; δ 7.24) and the signal of $CDCl_3$ (^{13}C-NMR, δ 76.9) were used as internal standards for all of the compounds with the exception of compounds 3 and 4, for which the internal standard for the ^{13}C-NMR was the signal of CD_3OD (δ 49.0).

Isolation of steroids by column chromatography. Individual steroid compounds were isolated from the culture liquid by column chromatography [28]. After 24 and 48 h of transformation, the contents of the flasks (250 mL) were extracted twice with an equal volume of ethyl acetate; the extract was evaporated to 2–2.5 mL at 40°C. Chromatography was performed on a column (16 × 450 mm) with silica gel 60 (Merck, Germany, 0.040–0.063 mm); hexane–ethyl acetate mixtures of different percentages were used for the elution.

RESULTS AND DISCUSSION

Identification of major products of DHEA transformation by *S. fumoso-rosea* VKM F-881. Four steroid compounds accumulated in the medium during DHEA transformation by the *S. fumoso-rosea* strain VKM F-881. These compounds were isolated by column chromatography and identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (Tables 1, 2, 3, and 4).

Peaks of molecular ions with a mass number of 304 were observed in the mass spectra of compounds 1 and 2. The spectra of both samples were almost identical; however, they differed in the intensity of the molecular ion peak in the spectrum of compound 1. Comparison of the data with the spectrum of the 7α-OH-DHEA standard demonstrated their agreement (Table 1). It was shown that the mass number of molecular ions as compared to DHEA increased by 16 units, and a peak with a mass number of 253, which is associated with the elimination of water molecules and two methyl groups from M^+304, was present in the spectra. This suggested that the analyzed compounds were products of DHEA oxidation and contained two hydroxyl groups in their structure.

The peak of molecular ion 320 was observed in the mass spectra of compounds 3 and 4. Both compounds had the same pattern of the mass spectrometric decomposition; it was primarily preconditioned by the

Table 2. Chemical shifts (δ, ppm) and SSCCs (J, Hz) in the ^{1}H-NMR spectra of the DHEA biotransformation products and model compounds (DHEA, 7α-OH-DHEA)

Compound	CH_3 (C-18)	CH_3 (C-19)	3-H*	6-H	7-H
DHEA (standard)	0.87 s	1.02 s	3.52 tt ΣJ = 32	5.36 dt $^3J_{6\text{-H, 7-Ha}} = 5.3$ $^3J_{6\text{-H, 7-Ha}} \approx 2.0$ $^4J_{4\text{-Ha, 6-H}} \approx 2.0$	–
7α-OH-DHEA (standard)	0.87 s	1.00 s	3.57 tt ΣJ = 32	5.63 dd $^3J_{6\text{-H, 7-H}} = 5.3$ $^4J_{4\text{-Ha, 6-H}} = 1.8$	3.96 m $^3J_{6\text{-H, 7-H}} = 5.3$ $^3J_{7\text{-H, 8-H}} = 3.5$ $^5J_{4\text{-Ha, 7-H}} \approx 2.0$
7β-OH-DHEA	0.88 s	1.06 s	3.54 tt ΣJ = 32	5.30 t $^3J_{6\text{-H, 7-H}} \approx 2.2$ $^4J_{4\text{-Ha, 6-H}} \approx 2.2$	3.94 tt $^3J_{6\text{-H, 7-H}} \approx 2.2$ $^3J_{7\text{-H, 8-H}} \approx 7.5 \approx {}^2J_{7\text{-H, 7-OH}}$ $^5J_{4\text{-Ha, 7-H}} \approx 2.2$
3**	1.32 s	1.01 s	3.55 tt ΣJ = 31.8	5.25 dd $^3J_{6\text{-H, 7-H}} = 2.6$ $^4J_{4\text{-Ha, 6-H}} = 1.9$	3.93 m $^3J_{6\text{-H, 7-H}} = 2.6$ $^3J_{7\text{-H, 8-H}} = 7.6$ $^5J_{4\text{-Ha, 7-H}} = 2.2$
4**	1.31s	0.95 s	3.57 tt ΣJ = 32	5.60 dd $^3J_{6\text{-H, 7-H}} = 5.4$ $^4J_{4\text{-Ha, 6-H}} = 1.8$	3.97 m $^3J_{6\text{-H, 7-H}} = 5.4$ $^3J_{7\text{-H, 8-H}} = 3.5$ $^5J_{4\text{-Ha, 7-H}} = 2.0$

* The multiplicity and S*J* are given without considering the interaction with the OH-group proton.
** The SSCCs were measured from the spectra obtained in $CDCl_3$ + CD_3OD.

Table 3. Comparison of the observed SSCCs for $^3J_{i-H,\ j-H}$ (Hz) with the values estimated using the dihedral angles j_{ij} between the planes of the bonds H–C_i–C_j and C_i–C_j–H in 7α-OH-DHEA and 7β-OH -DHEA isomers

Isomer	$^3J_{6\text{-H, 7-H}}/{}^3J^{estim*}_{6\text{-H, 7-H}}$	$\varphi_{6,7}$	$^3J_{7\text{-H, 8-H}}/{}^3J^{estim*}_{7\text{-H, 8-H}}$	$\varphi_{7,8}$
7α-OH-DHEA (standard)	5.3/6.32	50	3.5/7.5	37
2(7β-OH-DHEA)	2.2/2.4	70	7.5/10.0	160

* The SSCC values were calculated according to the Karplus equation using the following parameters: $A = 7$, $B = -1$, and $C = 5$.

elimination of H_2O and CH_3 groups from the molecular ion. The spectra only differed in the intensity of peak 284 $[M\text{-}2H_2O]^+$, suggesting that these compounds were isomers (Table 1).

As is shown in Table 2, it was not only the signals of the angular methyl groups that were identified in the ^{1}H-NMR spectra of the 7α-OH-DHEA standard and of compound 2; the signal of the proton from the H-6 double bond and signals in the region of δ 3.5-4.0 resulting from two protons geminal to the hydroxyl groups were also identified. Comparison of the spectra of the compound 2 and of DHEA, in which the signal was similar to the δ value and had analogous multiplicity, made it possible to relate the signal at δ 3.50 to a 3α-H proton. The position of the second of said signals on the δ scale was approximately the same in both samples (δ ~ 3.95), but their multiplicity varied greatly (Table. 2). Using double resonance, it was shown that

Table 4. Chemical shifts (δ, ppm) in the ^{13}C-NMR spectra of the DHEA biotransformation products (compounds 3 and 4) and the model compounds (DHEA, 7α-OH-DHEA, and 7β-OH-DHEA)

Carbon atom number	Compound				
	DHEA	7α-OH-DHEA	7β-OH-DHEA, 2	3*	4**
3	71.38	71.09	71.11	71.75	71.38
5	140.91	146.49	143.60	143.26	146.00
6	120.72	123.46	125.37	125.71	123.98
7	–	64.19	72.72	70.90	63.51
10	36.49	37.42	36.35	36.70	38.13
13	47.39	46.99	47.66	84.72	85.22
17	221.08	220.93	221.09	173.78	174.43
18	13.39	13.17	13.46	20.36[a]	20.28[b]
19	19.27	18.16	19.05	18.91[a]	18.47[b]

* The solvent was $CDCl_3 + CD_3OD$ (2 : 1, vol/vol), $\delta_{\underline{C}D_3OD}$ 49.00.

** The solvent was $CDCl_3 + CD_3OD$ (1 : 1, vol/vol), $\delta_{\underline{C}D_3OD}$ 49.00. a, b—a reverse assignment of the signals labeled by the same letter is possible.

the signal for both compounds matched a proton that interacts with 6-H with spin–spin coupling constants (SSCCs) of more than 2 Hz and doesn't interact with 3-H; thus, it was the signal of 7-H. Therefore, the 7α-OH-DHEA standard and compound 2 were isomers differing in the position of the OH group at C_7; it means that compound 2 is 7β-OH-DHEA. This assignment was confirmed by comparing the observed values of the vicinal SSCCs of $^3J_{6\text{-H}, 7\text{-H}}$ and $^3J_{7\text{-H}, 8\text{-H}}$ for two 7-hydroxy-isomers of 3β,7-dihydroxy-androst-5-en-17-one with the values calculated on the basis of the respective dihedral angles via molecular modeling of these isomers (Table 3). Further confirmation of the identification of compound 2 as 7β-OH-DHEA was the proximity of chemical shifts of the carbon atoms in the ^{13}C-NMR spectra of compound 2 and the 7α-OH-DHEA standard (Table 4).

A peak of the molecular ion M^+320 was observed in the mass spectra of compounds 3 and 4; it corresponded to an increase in the masses of the isomers described above, 7α-OH- and 7β-OH-DHEA, by one oxygen atom. Beside the 6-H signal, only two proton signals were observed in the ^1H-NMR spectra of compounds 3 and 4 at $\delta > 3.0$. These were the protons geminal to the OH group (as in the isomers described above, 7α-OH- and 7β-OH-DHEA). The choice between different possibilities of oxygen atom attachment to the steroid skeleton (by oxidation of tertiary or quaternary carbons) and of the formation of peroxide compounds was performed by comparing the ^{13}C-NMR spectra of compounds 3 and 4 with the model spectra of DHEA, 7β-OH-, and 7α-OH-DHEA (Table 4). Analysis of the data shown in Table 4 demonstrated that the number of primary, secondary, tertiary, and quaternary carbon atoms in the compounds **3** and **4** was equal to that in 7β-OH- and 7α-OH-DHEA. Thus, the chemical shifts of carbons in compounds 3 and 4 significantly differed from that in 7β-OH- and 7α-OH-DHEA. For instance, the C^{17} signal in compounds 3 and 4 was shifted upfield by about 50 ppm, and the C^{13} signal was shifted downfield by almost 40 ppm in comparison with the corresponding signals in 7β-OH- and 7α-OH-DHEA. The C^{18} signal also noticeably shifted downfield relative to 7β-OH- and 7α-OH-DHEA (and so did the $C^{18}H_3$ signal in the ^1H-NMR spectra (Table 2)) in compounds 3 and 4. All of these facts were indicative of the presence of an oxygen atom between C^{13} and C^{17} in compounds 3 and 4; that is, of a six-membered lactone structure of the D ring in these compounds. The structure of each of the lactones (com-

pounds 3 and 4) resulted from the comparison of the parameters of the ^{1}H- and ^{13}C-NMR spectra of these compounds with each other and with the spectra of DHEA, 7α-OH- and 7β-OH -DHEA (Tables 2 and 4). In the ^{1}H-NMR spectra, the 3-H signal multiplicity was equal in compounds 3 and 4 and agreed with that in DHEA and DHEA diols; this fact indicates that the C^3 center in all of these compounds has a 3β-OH configuration. The multiplicity of the second signal of the protons geminal to the OH group differed in compounds 3 and 4; however, in both compounds the signal corresponded to the 7-H proton, which interacted with 6-H. The similarity of the values of $^3J_{6\text{-H}, 7\text{-H}}$ and $^3J_{7\text{-H}, 8\text{-H}}$ for 7α-OH-DHEA and for compound 4, on one hand, and the similarity of these values for 7β-OH-DHEA and for compound 3, on the other, was reason to relate the C^7 configuration to compounds 3 and 4. A comparison of the C^7 chemical shifts in the ^{13}C-NMR spectra of the compounds (δ 70.90 and 63.51) with the chemical shifts of the same carbon atom in 7β-OH- and 7α-OH-DHEA (δ 72.72 and 64.19) confirmed the 7β-OH- and 7α-OH-DHEA configuration of compounds 3 and 4.

Therefore, compounds **3** and **4** were 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one and 3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one.

DHEA transformation by a strain of *S. fumoso-rosea* VKM F-881. The formation of hydroxylated lactones was observed after 24 h of DHEA (2 g/L) bioconversion by a strain of *S. fumoso-rosea* VKM F-881. After 48 h of bioconversion, the content of 3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one and 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one increased, while the content of produced 7α-OH- and 7β-OH-DHEA decreased. Assessment of the steroid content balance in the DHEA bioconversion medium showed the partial destruction of transformation products. It should be noted that the main product of the DHEA (2 g/L) bioconversion by *S. fumoso-rosea* VKM F-881 was 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one; its yield was 42%. The yield of another hydroxylated lactone—3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one—was much lower (13.7%).

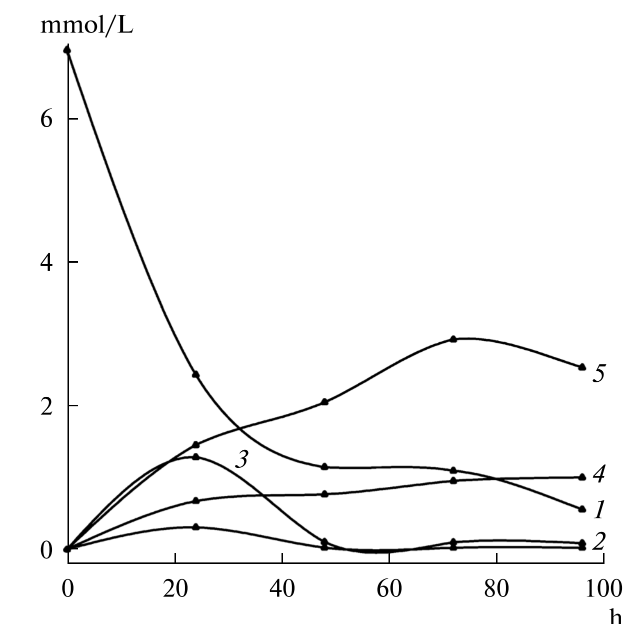

Fig. 1. Accumulation dynamics for the products of DHEA (2 g/L) transformation by a strain of *S. fumoso-rosea* VKM F-881. *1*—DHEA; *2*—7α-OH-DHEA; *3*—7β-OH-DHEA; *4*—3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one; and *5*—3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one.

The dynamics of accumulation and depletion of the DHEA transformation products suggested that, at the initial stage of the formation of steroid lactones, DHEA was hydroxylated at the C-7 position, producing 7(α/β)-OH-DHEA; further exposure of the product to BVMO resulted in the formation of the corresponding hydroxylated lactones. To confirm this assumption, we used 7α- and 7β-OH-DHEA as substrates. During incubation of the steroids (1 g/L) with *S. fumoso-rosea* VKM F-881, we observed the formation of the corresponding hydroxylated steroid lactones.

Table 5. DHEA transformation by *S. fumoso-rosea* VKM F-881

Substrate loading, g/L	Yield of hydroxylated lactones, mol %		Residual substrate, mol %	Transformation time, h
	3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one	3β,7α-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one		
5	63–70.2	16.2–18.9	4–5	46–48
10	67–72	19.8–22.5	1.5–3	96–120
20	49.5–54	11.2–15.6	1–5	120–144

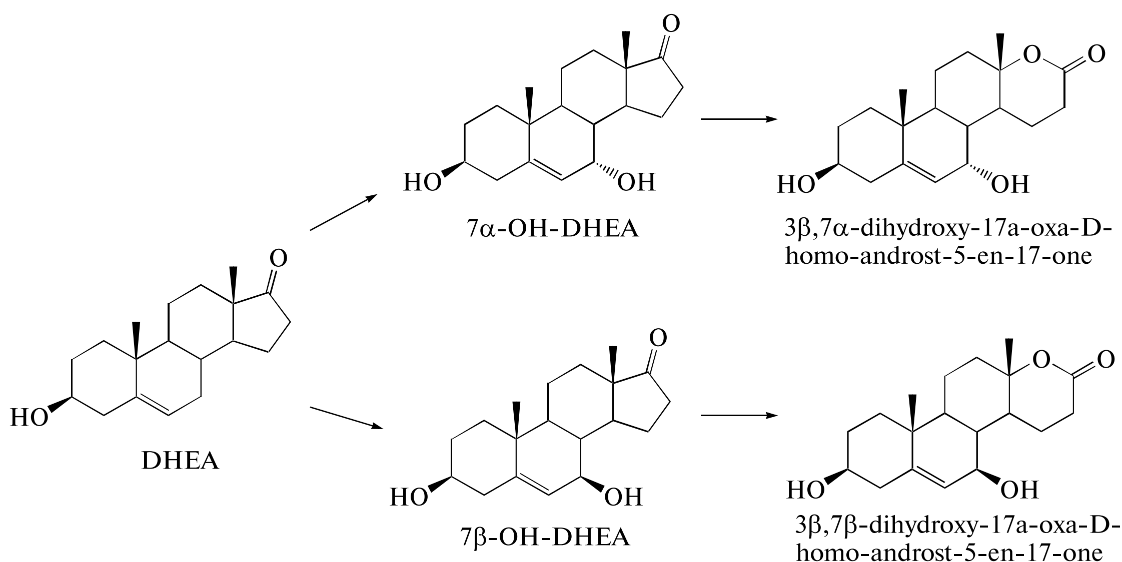

Fig. 2. Formation of steroidal lactones with a 3β-hydroxy-5-ene structure in DHEA transformation by the *S. fumoso-rosea* VKM F-881 culture.

According to the literature, the production of hydroxylated steroid lactones can be carried out by different pathways. For instance, in cultures *Penicillium lilacinum* [6], *Aspergillus tamarii* [5, 20], and *Fusarium javanicum* var. *ensiforme* [29], hydroxylated lactones were formed from steroid compounds, the structures of which already comprised the hydroxy group. Another pathway for the formation of the compounds is the microbiological hydroxylation of steroid lactones. For example, when transforming 3β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one by an *A. tamari* KITA culture, a series of saturated steroid lactones with the hydroxyl groups at the positions 1β, 6β, 7β, 11β, and 11α were obtained [30].

The obtained data on DHEA transformation by the *S. fumoso-rosea* strain VKM F-881 indicated that the formation of hydroxylated lactones resulted from the sequential 7(α/β)-hydroxylations of DHEA and the lactonization of the obtained derivatives at the D ring (Fig. 2).

To improve the efficiency of a microbiological process at high substrate loadings (5–20 g/L), we used richer and standardized growth and transformation media; the means of introducing the substrate into the bioconversion medium was also altered. These alterations made it possible to obtain hydroxylated lactones with a 3β-hydroxy-5-ene-structure with high yields (Table 5).

As can be seen from Table 5, the yield of the main product—3β,7β-hydroxy-17a-oxa-D-homo-5α-androstan-17-one—reached 72 mol % at a substrate loading of 5 g/L; at the initial DHEA concentration of 20 g/L, the yield was higher than 49%. The residual substrate content did not exceed 5% regardless of the initial DHEA concentration. The yield of 3β,7α-hydroxy-17a-oxa-D-homo-5α-androstan-17-one was in a range of 11.2–22.5 mol %. When the DHEA concentration in the medium was increased to 20 g/L, the transformation time increased to 120–144 h.

It is known that the yield of hydroxylated lactones in microbiological transformation is rather low, and the concentration of substrates never exceeded one gram per liter. For example, after DHEA (1 g/L) transformation by a strain of *A. tamari* KITA, several products were isolated, among which 3β,7α-dihydroxy-17-oxa-D-homo-androst-5-en-17-one was identified; its yield was 20.8% [19]. 3β,11α-Dihydroxy-17-oxa-D-homo-androst-5-en-17-one was obtained with a higher yield of 65% after DHEA transformation induced by a *B. bassiana* culture; however, the concentration of the substrate was only 0.23 g/L [18]. There is no data on the use of the genus *Spicaria* strains for microbial transformation of steroids and for Baeyer-Villiger reactions.

Thus, the possibility of the microbial steroid transformation using *S. fumoso-rosea* VKM F-881 was demonstrated. The strain can effectively produce 7(α/β)-hydroxylated lactones from DHEA in a single step by performing sequential hydroxylations and Baeyer-Villiger oxidation. At high substrate loading (5–20 g/L), the yield of 3β,7β-dihydroxy-17a-oxa-D-homo-androst-5-en-17-one was 49.5–72 mol %. It enabled us to evaluate the strain as promising for further practical use in the preparation of bioactive steroidal lactones required in clinical practice.

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REFERENCES

1. Peterson, D.H., Eppstein, S.H., Meister, P.D., Murray, H.C., Leigh, H.M., Weintraub, A., and Reineke, L.M., *J. Am. Chem. Soc.*, 1953, vol. 75, no. 22, pp. 5768–5769.
2. Fried, J., Thoma, R.W., and Klingsberg, A., *J. Am. Chem. Soc.*, 1953, vol. 75, no. 22, pp. 5764–5765.
3. Brannon, D.R., Martin, J., Oehschlager, A.C., Durham, N.N., and Zalkow, L.H., *Org. Chem.*, 1965, vol. 30, no. 3, pp. 760–762.
4. Zohri, A.A., *Folia Microbiol.*, 2000, vol. 45, no. 3, pp. 243–247.
5. Hunter, A.C., Elsom, J., Ross, L., and Barrett, R., *Biochim. Biophys. Acta*, 2006, vol. 1761, no. 3, pp. 360–366.
6. Sebek, O.K., Reineke, L.M., and Peterson, D.H., *J. Bacteriol.*, 1962, vol. 83, no. 6, pp. 1327–1331.
7. Bartmanska, A., Dmochowska-Gladysz, J., and Huszcza, E., *Steroids*, 2005, vol. 70, no. 3, pp. 193–198.
8. Kołek, T., Szpineter, A., and Swizdor, A., *Steroids*, 2008, vol. 73, no. 14, pp. 1441–1445.
9. Yang, B., Wang, Y., Chen, X., Feng, J., Wu, Q., and Zhu, D., *Tetrahedron*, 2014, vol. 70, no. 1, pp. 41–46.
10. Bartmanska, A. and Dmochowska-Gładysz, J., *Enzyme Microb. Technol.*, 2007, vol. 40, no. 6, pp. 1615–1621.
11. Plourde, R., El-Tayeb, O.M., and Hafez-Zedan, H., *Appl. Microbiol.*, 1972, vol. 23, no. 3, pp. 601–612.
12. Zhang, H., Ren, J., Wang, Y., Sheng, C., Wu, Q., Diao, A., and Zhu, D., *Tetrahedron*, 2013, vol. 69, no. 1, pp. 184–189.
13. Itagaki, E.J., *Biochemistry*, 1986, vol. 99, no. 3, pp. 825–832.
14. Miyamoto, M., Matsumoto, J., Iwaya, T., and Itagaki, E., *Biochim. Biophys. Acta*, 1995, vol. 1251, no. 2, pp. 115–124.
15. Donova, M.V. and Egorova, O.V., *Appl. Microbiol. Biotechnol.*, 2012, vol. 94, no. 6, pp. 1423–1447.
16. Swizdor, A., Panek, A., and Milecka-Tronina, N., *Steroids*, 2014, vol. 82, no. 1, pp. 44–52.
17. Kolek, T., Szpineter, A., and Swizdor, A., *Steroids*, 2009, vol. 74, nos. 10–11, pp. 859–862.
18. Swizdor, A., Kołek, T., Panek, A., and Białonska, A., *Biochim. Biophys. Acta*, 2011, vol. 1811, no. 4, pp. 253–262.
19. Hunter, A.C., Coyle, E., Morse, F., Dedi, C., Dodd, H.T., and Koussoroplis, S.-J., *Biochim. Biophys. Acta*, 2009, vol. 1791, no. 2, pp. 110–117.
20. McCurdy, J.T. and Garrett, R.D., *Org. Chem.*, 1968, vol. 33, no. 2, pp. 660–661.
21. US Patent No. 2744120, 1956.
22. Cella, J.A. and Kagawa, C.M., *J. Am. Chem. Soc.*, 1957, vol. 79, no. 17, pp. 4808–4809.
23. Budnick, R.M. and Dao, T.L., *Steroids*, 1980, vol. 35, no. 5, pp. 533–541.
24. Braunstein, G.D., *Endocr. Relat. Cancer*, 1999, vol. 6, no. 2, pp. 315–324.
25. Feuillan, P., Merke, D., Leschek, E.W., and Cutler, Jr. G.B., *Endocr. Relat. Cancer*, 1999, vol. 6, no. 2, pp. 303–306.
26. Seralini, G.-E. and Moslemi, S., *Mol. Cell. Endocrinol.*, 2001, vol. 178, nos. 1–2, pp. 117–131.
27. Lobastova, T.G., Gulevskaya, S.A., Sukhodol'skaya, G.V., and Donova, M.V., *Appl. Biochem. Microbiol.*, 2009, vol. 45, no. 6, pp. 617–622.
28. Lobastova, T.G., Gulevskaya, S.A., Sukhodolskaya, G.V., Turchin, K.V., and Donova, M.V., *Biocatal. Biotransform.*, 2007, vol. 25, no. 6, pp. 434–442.
29. US Patent No. 2823171, 1958.
30. Hunter, A.C. and Bergin-Simpson, H., *Biochim. Biophys. Acta*, 2007, vol. 1771, no. 9, pp. 1254–1261.

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