Synthesis of 3β-methyl ether of dehydroepiandrosterone by biotransformation of 3β-methyl ether of cholesterol with cells of mycobacteria *Mycobacterium sp*.

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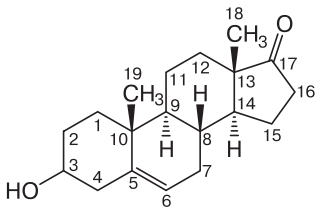
3β-Methyl ether of dehydroepiandrosterone was obtained by microbiological transformation of 3β-methyl ether of cholesterol with *Mycobacterium sp*. Androstane-3,17-dione, androst-4-ene-3,17-dione, and androsta-1,4-diene-3,17-dione were minor transformation products.

Key words: dehydroepiandrosterone, 3β-methyl ether of cholesterol, 3β-methyl ether of dehydroepiandrosterone, androstane-3,17-dione, androst-4-ene-3,17-dione, androsta-1,4-diene-3,17-dione, microbiological transformation, mycobacterium *Mycobacterium sp*., gas-liquid chromato-mass spectrometry.

Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) **1** is an endogenous steroid hormone possessing a wide range of physiological activity, for example, anti-inflammatory, antiproliferative, antidepressant, and neuroprotective.[1]



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Numerous literature data show that compound **1** has a protective effect against the development of obesity, cancer, diabetes, disorders of the adaptation process, memory, immune and cardiovascular systems, depression, Alzheimer's disease and other pathologies associated with aging.[1,2] In the human body, dehydroepiandrosterone **1** is the main intermediate in the synthesis of testosterone, estradiol, and androstenedione.[2] Recently, compound **1** was found to be the only source of sex steroids in women in the pre- and postmenopausal periods. It is also believed that various hormone-deficient symptoms are associated with a sharp age-related decrease in steroid **1** concentration.[3,4]

There are numerous data on the use of compound **1** in the treatment of diseases associated with age-related changes in hormonal levels.[5] In addition, steroid **1** and its metabolite (dehydroepiandrosterone sulfate) are widely used for the treatment and prevention of cardiovascular diseases.[6] There are known examples of its use in reproductology.[7] Compound **1** is also a valuable intermediate in the synthesis of drospirenone and a number of promising anticancer agents.[8]

The synthesis of dehydroepiandrosterone **1** from cholesterol by oxidation of dibromocholesterol acetate with chromic acid in acetic acid was first described[9] in the 1930s. It was widely used with small modifications even on an industrial scale for the synthesis of androgen hormones. However, this method is a multistage one, commercially inefficient, and environmentally unfriendly, since it produces a lot of waste.

It is known that compound **1** can be obtained by chemical modifications of androstenedione[10] or 16-dehydropregnenolone acetate (pregn-16-en-3β-ol-20-one acetate).[11] For a long time, the main starting material for the preparation of compound **1** was the 16-dehydropregnenolone acetate,[11] which is used for the industrial synthesis of androgens and corticoids. A six-step synthesis of steroid **1** from diosgenin with the intermediate generation of 16-dehydropregnenolone acetate in a 44% total yield is described in patent.[12]

Compound **1** also can be obtained by biotechnological methods. Thus, steroid **1** was obtained in no more than 6% yield[13] during transformation of cholesterol with resting *Pseudomonas convexa* cells. Also, dehydroepiandrosterone **1** can be obtained microbiologically using recombinant strains of *Mycobacterium smegmatis* with blocked enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$-isomer-

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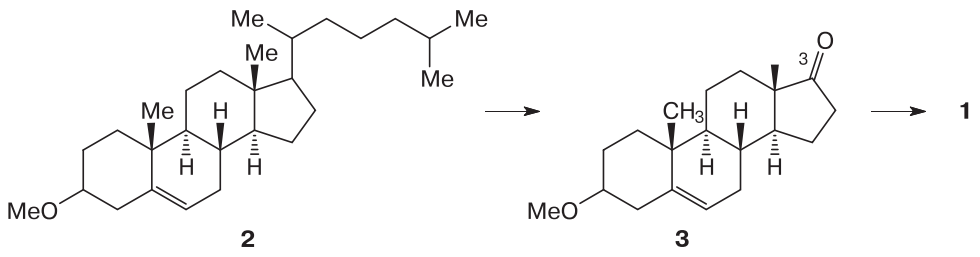
Scheme 1



ase[14,15] or by microbiological transformation of sterols of animal and plant origin, which include the stage of protection of the C(3) hydroxy group.[13,16] Since sterols can be considered as the most promising and affordable type of steroid raw materials (especially for Russia, where phytosterol can be obtained in unlimited amounts from forest waste), the option of microbiological synthesis of compound **1** from sterols becomes an actual issue.

Taking into account the above, it can be concluded that the use of cholesterol derivatives is promising for the preparation of compound **1**. We suggested a biotechnological method for its synthesis from 3β-methyl ether of cholesterol (**2**). The latter was transformed with the culture *Mycobacterium sp*. to 3β-methyl ether of dehydroepiandrosterone (**3**) (Scheme 1). Hydrolysis of ether **3** upon heating with dilute hydrochloric acid gave the target product **1**.[16]

Note that, within the framework of the approach involving microbiological preparation of dehydroepiandrosterone **1**, it is necessary to solve a number of key problems to successfully implement the biotransformation under consideration, namely, to efficiently eliminate the side chain while maintaining the Δ^5-bond and the hydroxy group at position C(3). In this case, one should take into account the high hydrophobicity of the ethers used in the transformation.[17]

In our work, we used bacterial cultures from the collection of the laboratory of physiologically active compounds of the Federal Research Center "Fundamentals of Biotechnology" of the Russian Academy of Sciences, *Mycobacterium sp*. R-77 and *Mycobacterium sp*. S-11094. The starting compound **2** (considering its high hydrophobicity) was used in a finely dispersed state. To improve the availability of the hydrophobic steroid substrate, we used Tween-80 and hydroxypropyl-β-cyclodextrin (HPCD) additives.

It was found that the mycobacteria are able to almost completely transform the starting compound **2** to form the main product **3** and a number of minor compounds, namely, androst-4-ene-3,17-dione (**4**), androsta-1,4-diene-3,17-dione (**5**), and androstane-3,17-dione (**6**) (Scheme 2).

Preliminary analysis was performed by TLC. The qualitative and quantitative composition of microbiological transformation products was determined by GLC-MS.

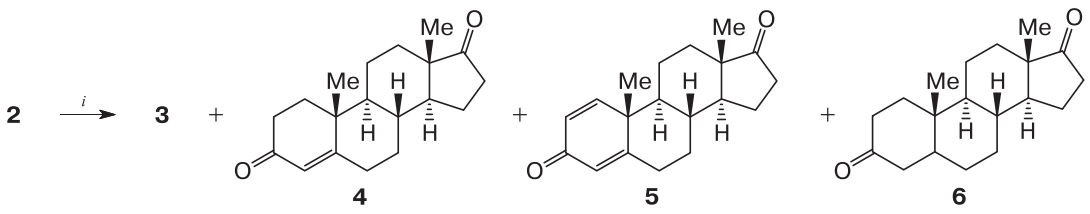
The target product **3** was isolated preparatively and characterized by physicochemical methods, which confirmed its chemical structure.

The data on the biotransformation of 3β-methyl ether of cholesterol (**2**) in the presence of Tween-80 are presented in Table 1.

As it is seen from Table 1, in the case of cell culture *Mycobacterium sp*. R-77 the content of the main product **3** after completion of the transformation was higher by 14.5%. Also, a lower content of the starting ether and byproducts in the final reaction mixture (a total of 22.5%) was observed as compared to the case when *Mycobacterium sp*. S-11094 was used (37%).

It is known that cyclodextrins are widely used in various industries as efficient solubilizers or stimulators of bioconversion processes. We used this technique in our previous works.[18,19] There is also literature data on the effect of cyclodextrins on the structure of the cell wall of microorganisms and, as a result, on the increase of the efficiency of bioconversion processes.[20] Therefore, it seemed advisable to study the effect of HPCD on the transformation rate of ether **2** and the accumulation of the

Scheme 2



i. *Mycobacterium sp*. S-11094 or R-77.

Table 1. Microbiological transformation of 3β-methyl ether of cholesterol (**2**) (2 g L^{-1} loading)

Culture used	Time/h	Conversion degree (%)	Relative content of steroids (%)				
			2	**3**	**4**	**5**	**6**
Mycobacterium sp. S-11094	92±2	92±2	2.7	63.0	33.6	0.7	—
Mycobacterium sp. R-77	92±2	95±2	1.1	77.5	1.2	20.2	Traces

Table 2. Effect of HPCD on the transformation of 3β-methyl ether of cholesterol (**2**) with cells *Mycobacterium sp*. R-77 (2 g L^{-1} loading)

Application form	Time/h	Conversion degree (%)	Relative content of steroids (%)				
			2	**3**	**4**	**5**	**6**
Finely dispersed suspension	92±2	94±2	4.2	77.6	18.0	0.2	Traces
In the presence of HPCD	88±2	97±2	0.3	81.5	17.5	0.5	0.2

main reaction products when using *Mycobacterium sp*. R-77. The results are presented in Table 2.

As it follows from Table 2, the use of HPCD in the transformation medium led to both a more complete conversion of the starting ether and a reduction in the transformation time. However, HPCD did not exert a critical effect on the transformation of 3β-methyl ether of cholesterol by *Mycobacterium sp*. R-77 at a 2 g L^{-1} loading.

In conclusion, mycobacteria *Mycobacterium sp*. efficiently transform 3β-methyl ether of cholesterol into 3β-methyl ether of dehydroepiandrosterone with minor admixtures of androst-4-ene-3,17-dione, androsta-1,4-diene-3,17-dione, and androstane-3,17-dione. It was found that the use of HPCD in the transformation medium leads to a more complete conversion of the starting ether **2** and a reduction in the transformation time.

Experimental

Transformation products were preliminary analyzed by TLC on Sorbfil plates (Imid Ltd, Russia).

Analytical study was performed on an Agilent Technologies instrument composed of a 7890 gas chromatograph (HP-5 column, 50 m × 320 μm × 1.05 μm) and a 5975 C mass selective detector with a quadrupole mass analyzer.

Preparative isolation of the target product was carried out on silica gel 0.060—0.200 mm, 40 Å (Acros, USA).

The melting point was measured in a capillary on an OptiMelt MPA-100 instrument (USA).

^{1}H NMR spectra were recorded on a Unity+400 spectrometer (Varian) with an operating frequency of 400 MHz.

Preparation of 3β-methyl ether of cholesterol (2). Methyl orthoformate (2.5 mL) and 57% $HClO_4$ (2 drops) were added to a suspension of cholesterol (5 g, 12.93 mmol) in anhydrous benzene (5 mL) with vigorous stirring. The reaction mixture upon stirring at 20 °C became entirely homogeneous and the reaction was completed within 2 h. The reaction progress was monitored by TLC. A 30% ethanolic solution of NaOH was added to bring the pH of the reaction medium to a neutral value, then water (50 mL) was added and the resulting mixture was extracted with benzene (50 mL). The benzene extract was concentrated under reduced pressure, the residue was additionally washed with acetone (20 mL) and dried. The dry residue (5.1 g) was dissolved in ethyl acetate (100 mL) at 20 °C with stirring. The undissolved precipitate was filtered off, the ethyl acetate filtrate was concentrated under reduced pressure, and the residue obtained was dried to obtain ether **2** (4.82 g, 90.4%), m.p. 86—87 °C (*cf*. Ref. 21: m.p. 81—82 °C). ^{1}H NMR ($CDCl_3$), δ (characteristic signals are only given): 0.70 (s, 3 H, C(18)H_3); 0.97 (s, 3 H, C(19)H_3); 3.08 (m, 1 H, H(3)); 3.36 (s, 3 H, OMe); 5.38 (br.s, 1 H, H(6)).

Microbiological transformation. The bacterial cultures *Mycobacterium sp*. R-77 and *Mycobacterium sp*. S-11094 were used in the work. The indicated bacterial cultures were stored on agar slants, the composition of which included the following components (g L^{-1}): glucose — 20.0, soy flour — 10.0, citric acid — 2.2, urea — 0.5, NH_4Cl — 1.0, KH_2PO_4 — 0.5, $MgSO_4$ — 0.5, $FeSO_4$ — 0.05, $CaCO_3$ — 1.5 (medium pH 6.8—7.2).

The biomass of mycobacteria aged 10—14 days obtained on the agar slopes was transferred into 750-mL conical flasks with 100 mL of the medium of the same composition and grew on a rocking shaker for 94—98 h at 28—30 °C with stirring at a rate of 220 rpm. Next, the seed material of mycobacteria in an amount of 20 vol.% was transferred into flasks equipped with chippers with a transformation medium of the following composition (g L^{-1}): glucose — 20.0, soy flour — 10.0, citric acid — 2.2, urea — 0.5, NH_4Cl — 1.0, KH_2PO_4 — 0.5, $MgSO_4$ — 0.5, $FeSO_4$ — 0.05, $CaCO_3$ — 1.5 (medium pH 6.8—7.2). The concentration of the starting compound (substrate) **2** (loading) was 2 g L^{-1}. Compound **2** was introduced into the reaction medium in the form of a finely dispersed suspension with the addition of Tween-80 (0.7 g L^{-1}) or HPCD (KLEPTOSE, France) in a 1 : 1 molar ratio. The flasks with the reaction medium were placed on a rocking shaker under conditions similar to these of growth.

The transformation products were analyzed by TLC (sorbent silica gel) and GLC-MS. To carry out the TLC analysis, we collected aliquots of the culture liquid (1 mL) at intervals determined

by the objectives of the experiment. Steroids were extracted with a four-fold volume of ethyl acetate or chloroform. Steroid compounds were separated by TLC, using a hexane—acetone solvent system (2 : 1) as the eluent.

To evaluate the residual starting sterols and their microbiological transformation products, the chromatograms were visualized with a 1% solution of vanillin in 10% $HClO_4$, followed by heating until colored spots appeared.

To isolate the products of transformation, the culture liquid (60 mL, with a loading of the starting substrate of 2 g L^{-1}) was extracted twice with an equal volume of ethyl acetate. Then, the ethyl acetate extract was concentrated under reduced pressure on a rotary evaporator. The oily residue obtained after evaporation of the solvent was dried in an oven at 60 °C.

To establish the structure of the main (major) product, the oily substance (50 mg) obtained after extraction and removal of the solvent was dissolved in $CHCl_3$ (1 mL) and applied to a preparative plate (sorbent silica gel; plate size 200×200 mm with a cut-out edge section 15×200 mm). A mixture of substances present in the isolated oily residue was separated by TLC (upstream version), eluting with the hexane : acetone solvent system (2 : 1). The cut-out section was visualized with a solution of vanillin in $HClO_4$ to localize the band of compound **3** on the chromatogram. The silica gel layer containing substance **3** was transferred from the chromatographic plate onto a No. 4 Schott filter and then washed with $CHCl_3$. The resulting solution was evaporated to dryness under reduced pressure on a rotary evaporator. Acetone was added to the residue to obtain a crystalline precipitate of compound **3**, which was filtered and dried at room temperature.

Product **3** has m.p. 136—137 °C (*cf.* Ref. 13: m.p. 139—140 °C). ^{1}H NMR ($CDCl_3$), δ (characteristic signals are only given): 0.87 (s, 3 H, C(18)H_3); 1.02 (s, 3 H, C(19)H_3); 3.08 (m, 1 H, H(3)); 3.35 (s, 3 H, OMe); 5.38 (br.s, 1 H, H(6)).

Gas-liquid chromato-mass spectrometry. The analytical samples collected from the corresponding reaction (transformation) mixtures were dissolved in ethyl acetate with stirring and shaking; the resulting suspension was centrifuged. The supernatant was decanted from the precipitate. The resulting solution was analyzed. The analysis was carried out without additional derivatization of the components.

The GLC-MS conditions are given below. The chromatography temperature program: an isotherm at 40 °C for 2 min; then programmed heating to 250 °C at 5 °C min^{-1}; an isotherm at 250 °C for 15 min; then programmed heating to 320 °C at 25 °C min^{-1}; an isotherm at 320 °C for 25 min; a splitless injector; injector temperature 250 °C; interface temperature 280 °C; carrier gas helium; flow rate 1 mL min^{-1}; chromatogram of samples by total ion current. Conditions for MS detection: energy of ionizing electrons 70 eV; registration of mass spectra in the positive ion mode in the m/z range from 20 to 450 at 2.5 scan s^{-1}; ChemStation E 02.00 software. The identification of the component composition (qualitative analysis) was carried out in accordance with the complete mass spectra database (NIST), as well as by comparison of the corresponding chromatographic retention times and chromatographic linear retention indices of tester compounds. The relative content (%) of components in the analyzed mixture (quantitative analysis) was calculated from the ratio of the areas under chromatographic peaks (by simple normalization). The results of the analytical study are presented in Tables 1 and 2.

Hydrolysis of 3β-methyl ether of dehydroepiandrosterone (3). A solution of 8% hydrochloric acid (200 mL) was added to the culture liquid containing compound **3** (100 mL). The mixture was stirred for 1 h at 80 °C. The completion of the reaction was determined by TLC analysis. Dehydroepiandrosterone **1** was isolated by extraction with chloroform. The chloroform extract was treated with activated carbon, the carbon was filtered off, the filtrate was evaporated *in vacuo*. Acetone was added to the crystalline residue, compound **1** was collected by filtration, the precipitate was dried at 60 °C to obtain compound **1**, m.p. 142—144 °C (*cf.* Ref. 13: m.p. 140—141 °C).

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