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Optimization of the Method of Obtaining 9α-Hydroxy-androst-4-ene-3,17-dione—the Key Intermediate in the Synthesis of Highly Active Fluorinated Corticosteroids from Phytosterols

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Abstract—A method of phytosterol conversion into 9α-hydroxy-androstenedione under high loads of steroid substrate has been developed with the use of mixed cultures of the actinobacteria *M. neoaurum* and *R. erythropolis*. The introduction of a 9α-hydroxylating culture after the first 70 h of conversion made it possible to increase process selectivity and to exclude the use of methylcyclodextrin, which is expensive and difficult to regenerate.

Keywords: phytosterols, mixed cultures, 9α-hydroxylation, fluorinated corticosteroids

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INTRODUCTION

At present, phytosterols (PS) are the cheapest and most promising source of raw material for the synthesis of pharmaceutical steroids. In a previous work [1], we developed an effective method of PS isolation and purification from a byproduct of soybean oil production. In addition, we proposed original methods to cleave the sterol side chain to 17-ketoandrostans: androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD) and 9α-hydroxy-AD (9α-OH-AD), which are key intermediates for the production of nearly all steroid drugs [2–10]. It is considered that ADD is a perfect intermediate for the synthesis of estrogens, anabolic steroids, and gestagenic 19-norsteroids and that AD is a perfect intermediate for the synthesis of sex hormones (androgens and pregnane gestagens) [11–14], while 9α-OH-AD is believed to be the most convenient intermediate for the industrial production of a new generation of fluorinated corticoids based on a modified steroid molecule with fluorine atoms at C9 and C6 and in the dioxyacetone chain, such as triamcinolone, dexamethasone, betamethasone, fluocinolone, mometasone, and fluticasone, which possess high anti-inflammatory, antiallergic, immunosuppressive, and antishock activities with a minimum of adverse effects [15–18]. The use of 9α-OH-AD as a key intermediate provides the shortest and easiest way to obtain fluorinated analogs of steroids; it makes it possible to eliminate the labor-intensive and noneconomic stage of microbiological 11-hydroxylation from the production process due to the easily implementable dehydration of the 9α-OH group.

At present, 9α-OH-AD is obtained from animal and plant sterols by two methods. One method includes a two-stage process of AD production from sterols via microbiological transformation by bacteria of the genus *Mycobacterium* [19–21], followed by 9α-phydroxylation of the formed AD by bacterial cultures [4, 7, 8, 20, 22]. The other method includes sterol conversion to 9α-OH-AD by mutant microbial strains with blocked synthesis of 1,2-dehydrogenase [23–25]. Thus, 9α-OH-AD can be obtained by successive exposure of a steroid molecule to different microorganisms with isolation of intermediate products, by exposure to successive microbial transformation without the isolation stage [26], or by the inclusion in this process of mixed microbial cultures. For the latter two cases, it is necessary that the used transformer microorganisms were not antagonists [27, 28].

A mixture of two microorganisms was used for steroid synthesis for the first time in 1939 [27]. As a result of AD transformation by cultures of *Bacillus putrificus* and *Saccharomyces cerevisiae*, both the keto groups and the double bond were reduced with the formation of 5α-androstan-3β,17β-diol [27]. The effects of the bacteria *Corynebacterium equi* and *Trichomonas foetis* on AD allows single-stage introduction of a Δ^1-bond and reduction of a 17-keto-group to 1,2-dehydrotestosterone. In addition, AD transformation by mixed

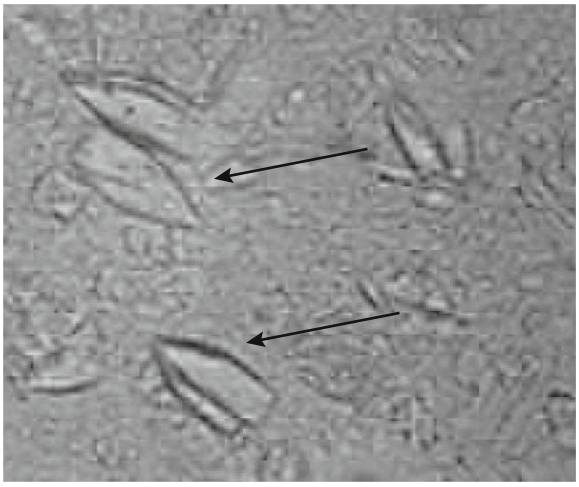

Fig. 1. AD crystals in the culture liquid of *M. neoaurum*.

cultures of *Fusarium lateritium* and *Saccharomyces cerevisiae* resulted in the formation of 1,2-dehydrotestosterone (with a 60% yield) [27]. 9α-fluorohydrocortisone was transformed into Δ^1-dehydro-16α-hydroxyfluorohydrocortisone (triamcinolone) with mixed cultures of *Arthrobacter simplex* and *Streptomyces roseochromogenus*. The reactions of 1,2-dehydration and 16α-hydroxylation were performed by *A. simplex* bacterial and *S. roseochromogenus* fungal cultures, respectively [28].

The microbiological transformation of steroids by mixed cultures simplifies the technological process and decreases its cost due to the absence of additional isolation and purification stages of intermediate products.

The goal of the work was to improve the method for obtaining 9α-OH-AD from PS, which is to be used in the industrial production of fluorinated corticosteroids.

MATERIALS AND METHODS

Microorganisms. The following microbial strains were used in the work: *Mycobacterium neoaurum* Ac-1634, which cleaves the side chain of animal and plant sterols with the formation of AD [2], and *Rhodococcus erythropolis* Ac-1740, which performs 9α-hydroxylation of Δ^4-3-ketosteroids [4, 29]. All of the stains were deposited at the Russian National Collection of Industrial Microorganisms (VKPM).

Media and cultivation conditions. The actinobacteria were stored on agar media. Medium I (pH 6.8–7.0) for *M. neoaurum* contained (g/L) the following: glucose, 10.0; soybean meal, 5.0; citric acid, 2.2; urea, 0.5; NH_4Cl, 1.0; KH_2PO_4, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.05; and $CaCO_3$, 1.5. Medium II (pH 6.8–7.2) for *R. erythropolis* contained (g/L): glucose, 10.0; maize extract, 15.0; and K_2HPO_4, 1.0.

M. neoaurum biomass (5- to 7-day) washouts from agar slants were put into 750-mL cone flasks with 100 mL of medium I and grown for 90–96 h on a shaker with stirring at 220 rpm and 28°C. The *M. neoaurum* inoculum was then concentrated two times under sterile conditions.

R. erythropolis biomass (4- to 5-day) washouts from agar slants were put into 750-mL cone flasks with 100 mL of medium II and grown for 70–72 h on a shaker with stirring at 220 rpm and 28°C.

The resultant *R. erythropoli*s inoculum (10% (vol.)) was transferred into analogous flasks with medium II and grown for 24 h under the conditions described above.

The *R. erythropolis* biomass for transformation stage 2 was settled for 24 h at 4–5°C. The *R. erythropolis* inoculum was then concentrated two times under sterile conditions.

The bacterium *M. neoaurum* was cultivated for the stage of sterol conversion to AD in analogous flasks with a baffle plate in medium III, pH 7.0–7.2, containing (g/L) the following: glucose, 20.0; 18%-fat soybean meal, 20.0; citric acid, 3.0; urea, 0.65; $(NH_4)_2HPO_4$, 2.0; $MgSO_4$, 0.65; $FeSO_4$, 0.065; $CaCO_3$, 3.0; and PS with a particle size of 5–15 μm, 30.0; pH 7.0–7.2. The glucose solution and inoculum were fed by fractions: 20%, 0 h; 10%, after 48 h; 10%, after 96 h.

Concentrated *R. erythropolis* biomass in an amount corresponding to 0.4–0.5 g of dry substance was placed into flasks with 100 mL of culture liquid obtained after 70 h of PS transformation by the *M. neoaurum* culture.

Quantification of transformation products. The amount of AD and 9α-OH-AD in the culture liquid was assayed by TLC. Steroids were extracted with ethyl acetate. Sorbfil plates (Imid Ltd., Russia) and a benzene–acetone (3 : 1) system were used for the analysis. Culture liquid aliquots (1 mL) were taken at intervals determined by the purposes of the experiment. Steroids were extracted from the samples with a 4-fold volume of ethyl acetate. The steroid compounds were separated in a benzene/acetone solvent system (3 : 1). The quantification of bioconversion products in the samples was performed visually in UV light with a XL-6 chromatoscope (Russia) via a comparison of the intensity of spot absorption at 254 nm with the spots of standards of known concentrations.

Isolation. The steroids were extracted three times by equal volumes of ethyl acetate from 200 mL of the culture liquid of 6 g of 95% PS. The combined extracts were clarified with 1 g of activated carbon with stirring for 15 min. The carbon was filtered, and the filtrate was evaporated in a vacuum. The resultant precipitate was treated with 10 mL of diethyl ether with stirring for 30 min. The precipitate was filtered, washed with diethyl ether, and dried. The yield was 2.69 g of 9α-OH-AD (the yield of 64.3% per PS).

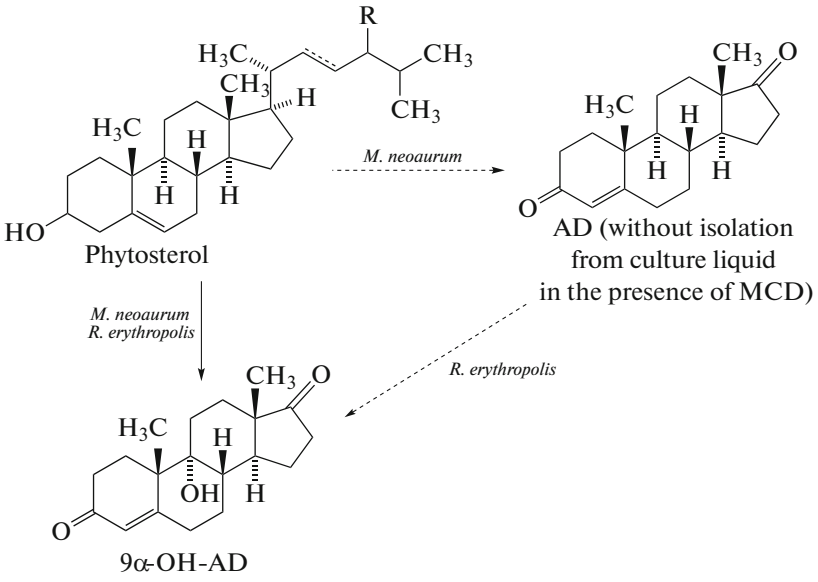

Fig. 2. Scheme of obtaining 9α-OH-AD from PS with and without MCD.

RESULTS AND DISCUSSION

In a previous work [7], we described the developed, improved method to obtain 9α-OH-AD from PS via the combined cultivation of actinobacteria *M. neoaurum* and *R. erythropolis*, in which PS was transformed to 9α-OH-AD by mixed cultures in two successive stages without isolation of the formed AD from the culture liquid at the first stage.

The technique of mixed cultures made it possible to simplify the technology of steroid substance production and to reduce its cost. However, the AD accumulated in the form of large crystals at the first stage after PS side chain cleavage with enhanced load (30 g/L or more) (Fig. 1), and it was many times larger than bacterial cells; thus, it became difficult to produce for further transformation to 9α-OH-AD.

Methyl-β-cyclodextrin (MCD), which was used for PS transformation at a weight ratio of 1 : 5 in order to solve this problem, formed a water-soluble inclusion complex with AD generated during sterol cleavage, followed by AD conversion to 9α-OH-AD (Fig. 2) [7].

However, MCD is an expensive reagent that is difficult to regenerate; therefore, later attempts were made to obtain an analogous yield without the use of MCD but with the same substrate load.

To prevent the enlargement of the formed AD crystals, which depends to a significant degree on the steroid concentration, the 9α-hydroxylating culture of *R. erythropolis* was added in 70 h of transformation. This corresponded to the exponential culture growth phase of *M. neoaurum* and intensive AD accumulation (Fig. 3a). The crystal structures that formed in the culture liquid after reaching an AD concentration of more than 10 g/L (Fig. 1) were inaccessible for further introduction of hydroxyl group at the 9α-position of steroid molecule, which is catalyzed by the *R. erythropolis* culture.

The microbiological transformation was intensified by fractional administration of the inoculum and

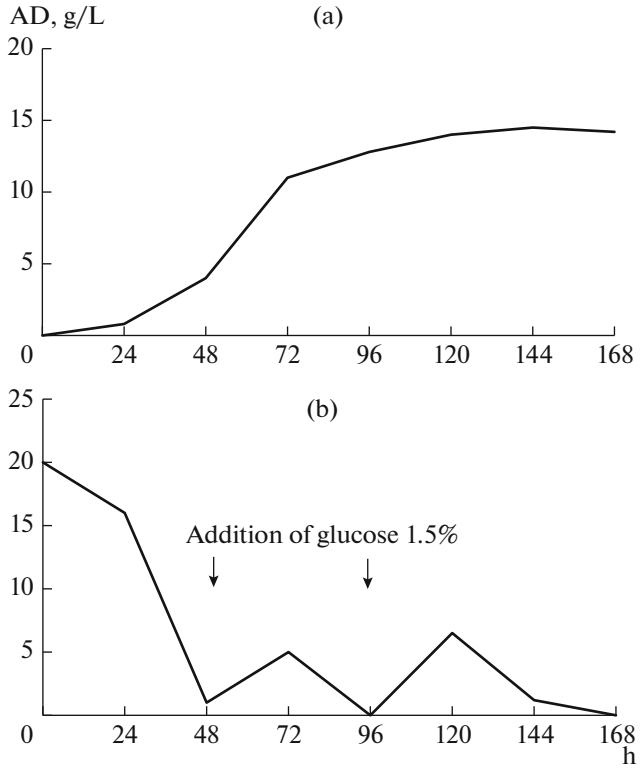

Fig. 3. Dynamics of AD accumulation and glucose uptake during PS conversion (30 g/L) by *M. neoaurum* culture.

glucose solution. As one can see from Figure 3b, the glucose reserve proved to be completely exhausted after 48 and 96 h of bioconversion and, therefore, glucose in the amount of 1.5 vol % and inoculum *M. neoaurum* (10 vol %).

Thus, due to the addition of *R. erythropolis* after 70 h of transformation, the formed AD was immediately hydroxylated to 9α-OH-AD. The transformation time at a PS load of 30 g/L in the absence of solubilizing agents was 168 h and the 9α-OH-AD yield was 64–65%.

In addition to the reduced process cost due to the absence of expensive MCD, an advantage of this method may be its high selectivity without the formation of byproducts, while the minor amounts of testosterone that form as a byproduct are hydroxylated by *R. erythropolis* with the formation of 9α-OH-AD [8].

CONCLUSIONS

Thus, we have developed an efficient method of PS transformation to 9α-OH-AD with the involvement of mixed cultures of actinobacteria *M. neoaurum* and *R. erythropolis* at high loads of steroid substrate.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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