HYDROXYLATION OF ANDROST-4-ENE-3,17-DIONE WITH THE AID OF *Curvularia lunata* FUNGUS

S. D. Shuvalova,[1] K. N. Gabinskaya,[1] E. V. Popova,[1] T. S. Savinova,[1] and V. A. Andryushina[1]

Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 35, No. 5, pp. 44 – 46, May, 2001.

Original article submitted November 30, 2000.

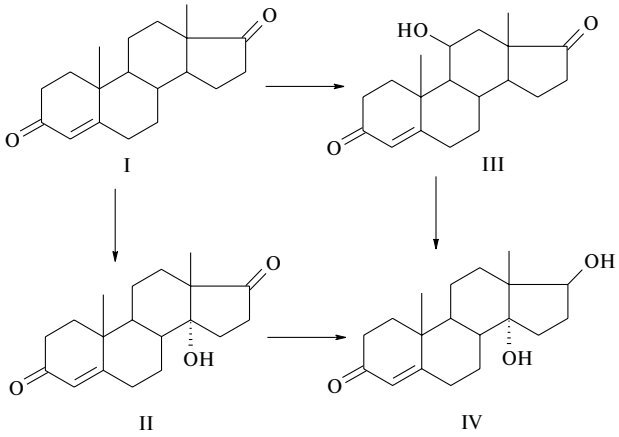
As is known, *Curvularia lunata* fungus is capable of introducing a hydroxy group at the 11β position of steroidal molecules [1] or at the 14α position of compounds of the androstane series [2, 3]. The process of 11β-hydroxylation of pregnanes (for example, of cortexolone and its derivatives), which is widely used in the commercial synthesis of corticosteroid drugs, has been studied in sufficient detail. In particular, it is known that the target product yield may be accompanied by the formation of up to 10% of a 14α-hydroxy compound [1].

In contrast to the hydroxylation of pregnanes, the analogous transformation of compounds of the androstane series by *Curvularia lunata* fungus has been studied to a much smaller extent, although development of a method for obtaining 14α-hydroxy androstane derivatives is of practical interest for the synthesis of highly effective steroidal preparations [3].

Below we present the results of investigation of the hydroxylation of androst-4-ene-3,17-dione (AD, I) with the aid of *Curvularia lunata* fungus. The purpose of this study was to select an optimum transformation regime, isolate and identify the possible side products, and determine the conditions of formation of these products. The AD transformation was studied using a *Curvularia lunata* fungal culture from the collection of the Bioengineering Center (Russian Academy of Sciences, Moscow). The process was performed using mycelium washed from a growth medium.

The study of the AD hydroxylation by *C. lunata* showed that the process is nonselective. In addition to the target product (14α-hydroxyandrost-4-ene-3,17-dione, II), the fungus yields a mixture of several other substances, from which we isolated and identified (by thin-layer silica gel chromatography) 11β-hydroxyandrost-4-ene-3,17-dione (III) and 14α,17β-dihydroxyandrost-4-en-3-one (IV).

An increase in the time of AD transformation (at a concentration of 1.5 g/liter) from 40 to 66 h led to a decrease in the concentration of compound II and an increase in the amount of compound IV (Table 1), while the content of compound III in the mixture exhibited no significant variations. The change in the ratio of fermentation products II and IV with increasing process duration can be explained by the formation of IV from II in the presence of 17-ketoxide reductase. Although the process of 17-keto group reduction has been described for some other microbiological cultures [4, 5], it is interesting to note the presence of this stage in the process conducted by *C. lunata*.



The mycelium of *C. lunata* grows in a liquid culture medium up to 1 g/100 ml. This mycelium has a creamy color and possesses a high hydroxylating activity when transferred monthly by separate colonies to agar medium. If the transfer period is increased, the mycelium grows up to 2 g/100 ml, exhibiting a black color and low hydroxylating activity. The mycelium was grown in two media: the first inoculate was grown in medium A and the second, in medium H. Both media provided for a high activity of the grown mycelium.

The amount of mycelium involved in the transformation was varied from 0.7 to 1.8 g/100 ml. At a substrate concentration below 4 g/liter, the optimum biomass concentration was 1 g/100 ml; at a greater steroidal load, the optimum mycelium content increases to 1.2 g/100 ml, but further increase in the biomass concentration is inexpedient.

[1] Bioengineering Center, Russian Academy of Sciences, Moscow, Russia.

The transformation process was performed in a phosphate buffer (pH 6.2), physiological solution, and 0.5% glucose solution. For an initial steroid concentration below 4 g/liter, these media proved to be equivalent. At a greater steroidal load, the transformation time increases from 48 to 120 h (Table 1); conducting the process in glucose solution hinders autolysis of the mycelium.

The initial substrate was introduced into the transformation medium by various methods: (i) DMF solution; (ii) solution in methanol containing 10% $CaCl_2$; (iii) dry powder ground in a mortar. It was found that DMF inhibits the hydroxylation process; the two other methods were equivalent (Table 2). Introduction of a surfactant (Sorbital S 20) at a concentration of 10 – 30 mg% together with the substrate did not increase the yield of hydroxylated product II (Table 2).

Thus, investigation of the process of AD hydroxylation by the washed mycelium of *C. lunata* fungus showed that the transformation is accompanied by the formation of side products (11β-hydroxyandrost-4-ene-3,17-dione and 14α,17β-dihydroxyandrost-4-en-3-one). Optimum hydroxylation conditions were established under which the target product 14α-hydroxyandrost-4-ene-3,17-dione is obtained with a yield of 55% (see the experimental part).

EXPERIMENTAL PART

The TLC analysis of compounds I – IV was performed on Silufol UV-254 plates (Czech Republic) eluted in a methylene chloride – acetone (7 : 3) system; he spots were developed by 1% vanillin solution in 10% aqueous hydrochloric acid. The IR and UV absorption spectra were recorded on a Specord M-80 and Specord UV-VIS spectrophotometers, respectively (Germany). The mass spectra were obtained with a Finnigan SSQ-710 spectrometer (USA) with a system of direct sample injection into the ion source operated at an electron beam energy of 70 eV. The ^{1}H NMR spectra were measured on a Unity Plus 400 spectrometer (Varian, USA) with a working frequency of 400 MHz. Quantitative analysis of the reaction mixtures was performed by HPLC in a Gilson chromatograph (France) using C-18 columns.

Culture growth and substrate transformation. The culture of *Curvularia lunata* fungus from the collection of the Bioengineering Center (Russian Academy of Sciences, Moscow) was maintained in tubes with agar medium. Medium composition (%): glucose, 0.4; yeast extract, 0.4; malt extract, 1; agar, 2.5 (pH 6.2). The mycelium was grown in media H and A; medium H (%%): saccharose, 2; microbial biomass inoculate, 0.25; sodium nitrate, 0.2; dibasic ammonium phosphate, 0.3; monobasic potassium phosphate, 0.1; potassium chloride, 0.05; magnesium sulfate, 0.05 (pH 6); medium A (%%): glucose, 2; soybean meal, 0.5; yeast extract, 0.5; monobasic potassium phosphate, 0.5; sodium chloride, 0.5 (pH 6.5).

14*α*-Hydroxyandrost-4-ene-3,17-dione (II). An aqueous suspension of fungal spores (2 ml) was introduced into 750-ml flasks containing 100 ml of medium A. The first inoculate was grown for 48 h. This seeding material (3 ml) was introduced into flasks with medium H and the second inoculate was grown for 36 h. Mycelium was filtered from the medium, washed with water, and transferred into 100 ml of 0.5% glucose solution (for other media, see the text above).

The main transformation product II was accumulated by conducting the process at a substrate load of 4 g/liter. After a 72-h incubation, the cultural liquid with mycelium was extracted with ethyl acetate. After evaporation, the oily residue was crystallized from ether to isolate compound II; yield 55%; m.p., 247 – 249°C (reported data: m.p., 252 – 254°C [8]).

The side transformation products were isolated from mother liquor solutions in ether combined after several runs. The oily residue (8.2 g) obtained upon solvent evaporation was chromatographed on a column filled with 100 g silica gel 40/100 μ to isolate compounds II – IV.

11β-Hydroxyandrost-4-ene-3,17-dione (III). Compound III is eluted first with chloroform; yield 0.56 g (6.8%); m.p., 185 – 187°C; IR spectrum (ν_{max}, cm^{-1}): 3400 (OH), 1650 (CO), 1740 (CO), 1610 (C=C); ^{1}H NMR spectrum in $CDCl_3$ (δ, ppm): 1.139 (s, 3H, 18-CH_3), 1.441 (s, 3H, 19-CH_3), 4.437 (m, 11-H), 5.674 (d, 4-H); mass spectrum, *m/z*: 302, 284, 269, 189, 163, 124, 123; reported data: m.p., 183 – 187°C [6]; mass spectrum, *m/z* (I_{rel}): 302 (86), 284 (19), 269 (23), 189 (42), 163 (100), 124 (76), 123 (82) [7].

TABLE 1. Effect of Androst-4-ene-3,17-dione Transformation Conditions on Product Composition (HPLC Data)

Batch No.	Substrate concentration, g/liter	Process time, h	Relative product yield, %		
			II	III	IV
1	1.5	40	69.4	6.7	15.7
2	1.5	66	52.4	6.5	24.7
3	2.0	48	53.4	6.1	16.9
4	4.0	48	59	7	16.0
5	4.0	72	66.8	6.8	18.5
6	6.0	120	60	6.5	23.8

TABLE 2. Effect of the Method of Androst-4-ene-3,17-dione (I) Introduction on the Yield of 14α-Hydroxylated Derivative II

Form of I	Surfactant (Sorbital S 20) additive	Yield of II, %
Ground I	–	52
Ground I	+	55
DMF solution	–	30
Methanol solution with 10% $CaCl_2$	–	58

14*α*-Hydroxyandrost-4-ene-3,17-dione (II). Compound II is eluted second with chloroform – 20% acetone mixture; yield 1.18 g (14.4%); m.p., 252 – 255°C; UV spectrum (λ_{max}, nm): 242 (ε, 15,900); reported data [8]: m.p., 252 – 254°C; UV spectrum (λ_{max}, nm): 240 (ε, 15,800).

14*α*,17β-Dihydroxyandrost-4-en-3-one (IV). Compound IV is eluted last with a chloroform – methanol (9 : 1) mixture; yield 1.52 g (18.5%); m.p., 193 – 195°C; mass spectrum, *m/z*: 304, 286, 271, 165, 126, 125; IR spectrum (ν_{max}, cm^{-1}): 3600 (OH), 1670 (CO), 1620 (C=C); reported data [9]: m.p., 183.5 – 186°C; IR spectrum (ν_{max}, cm^{-1}): 3467 (OH), 1649 (CO), 1611 (C=C).

REFERENCES

1. G. V. Sukhodol'skaya, B. A. Angelova, and K. A. Koshcheenko, *Prikl. Biokhim. Mikrobiol.*, **22**(2), 226 – 237 (1986).
2. L. A. Krasnova, O. V. Messinova, V. I. Bayunova, et al., USSR Inventor's Certificate No. 801517 (1979); *Chem. Abstr.*, **110**, 133697u (1989).
3. Ger. Offen. 4.129.005 (Cl. C 07 J 1 / 00) (1993); *Chem. Abstr.*, **118**, 190112 p (1993).
4. K. E. Smith, S. Latif, and D. N. Kirk, *J. Steroid Biochem.*, **32**, 445 (1989).
5. R. Krishman, K. M. Madyastha, and M. A. Vismamitra, *Steroids*, **56**, 440 (1991).
6. R. M. Dodson, S. Kraychy, et al., *J. Org. Chem.*, **27**, 3159 – 3164 (1962).
7. S. S. Simons, J. Merchlinsky, and D. F. Idinson, *Steroids*, **37**(3), 281 – 289 (1981).
8. K. Singh, S. N. Sehgal, and C. Vizina, *Canad. J. Microbiol.*, **13**, 1271 (1967).
9. S. H. Eppstein, P. D. Meister, D. H. Peterson, et al., *J. Am. Chem. Soc.*, **80**, 3382 – 3389 (1958).