MICROBIOLOGICAL HYDROXYLATION OF STEROIDS I.
MICROBIOLOGICAL HYDROXYLATION OF PROGESTERONE
BY RHIZOPUS

TOSHI NOBU ASAI, KYOSUKE TSUDA, KÔ AIDA, EIJII OHKI,
TOKUJI TANAKA, MASAKO HATTORI and
HARUO MACHIDA

The Institute of Applied Microbiology, and Department of Agricultural
Chemistry, Faculty of Agriculture, University of Tokyo

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In recent years, a new synthetic process(1) to introducing a substituent
into the steroid skeleton by means of enzymatic hydroxylation of microorga-
nisms for the preparation of adrenocortical hormones whose chemical synthesis
would otherwise be extremely difficult and require a long process has been
established. It has now become possible to introduce a hydroxyl group into
the 11-position of steroids such as progesterone, cortexone, and compound S
to form 11-hydroxy- and 11-oxocorticosteroids by the use of microorganisms
such as Rhizopus(2) and Aspergillus(3).

Before going into this study, the capability of such hydroxylation was
tested with 284 strains of Rhizopus sp. preserved in this Institute, using
progesterone. As it will be described in the experimental section, the micro-
organism was incubated, the fermentation liquor was extracted, and the
concentrated extract examined by paper chromatography, following the
method of Zaffaroni(4). It was thereby found the 21 strains of this species,
listed in Table 1, had the ability to effect this hydroxylation. Further, mass-
culture was carried out on a few strains, the products isolated and purified

\[ \text{CH}_3 \]
\[ \text{CO} \]
\[ \text{O} \]
\[ \text{RO} \]

(I) \( R = H \)

\[ \text{CH}_3 \]
\[ \text{CO} \]
\[ \text{O} \]

(II) \( R = \text{COCH}_3 \)

(III)
by chromatography, upon which hydroxylation product was identified to be 11α-hydroxyprogesterone (I). Its physical constants, its acetate (II), and formation of 11-oxoprogesterone (III) by further oxidation, all agreed well with those reported in previous literature\(^6\).

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**EXPERIMENTAL**

1. Examination of the Hydroxylation Ability of Each Strain.

The medium used here for selection of the strain contained 5% glucose, 2% peptone, and 0.3% corn steep liquor. Ten ml of this medium was placed in each test tube of 35 ml capacity, (17×240 mm) previously cotton plugged and

<table>
<thead>
<tr>
<th>Species</th>
<th>No</th>
<th>Hydroxylation ability</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rh. nigricans</em> YAMAZAKI</td>
<td>R5-4</td>
<td>+++</td>
<td>11α-Hydroxyprogesterone (identified)</td>
</tr>
<tr>
<td><em>Rh.</em></td>
<td>R7-7</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td><em>Rh. chinensis</em> SAITO</td>
<td>10-10</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td><em>Rh. chiniiang</em> YAMAZAKI</td>
<td>14-14b</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. arrhizus</em> FISCHER</td>
<td>R5-6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. Kansho</em> YAMAMOTO</td>
<td>28-14</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. nigricans</em> EHRENBERG</td>
<td>37-12</td>
<td>+</td>
<td>11α-Hydroxyprogesterone (by paperchromatography)</td>
</tr>
<tr>
<td><em>Rh.</em></td>
<td>37-27</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. delemar</em> WEHMER et HANZAWA</td>
<td>19-27</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. formosensis</em> NAKAZAWA</td>
<td>21-29c</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. japonicus</em> VUILLEMIN</td>
<td>24-14</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh.</em></td>
<td>24-14b</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. javanicus</em> TAKEDA</td>
<td>26-23</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. nodosus</em> NAMYSLOWSKI</td>
<td>39-29a</td>
<td>+</td>
<td></td>
</tr>
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<td><em>Rh. pseudochinensis</em> YAMAZAKI</td>
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<td></td>
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<tr>
<td><em>Rh. tonkinensis</em> VUILLEMIN</td>
<td>65-10</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td><em>Rh. tritici</em> SAITO</td>
<td>66-5a</td>
<td>+</td>
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</tr>
<tr>
<td><em>Rh.</em></td>
<td>66-14c</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh.</em></td>
<td>66-27</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. niveus</em> YAMAZAKI</td>
<td>R5-5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Rh. oryzae</em> WENT et PRINS-GEERLIGS</td>
<td>R5-7</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
sterilized, and sterilized at 15 lb for 10 minutes (pH 5.2). The seed cultured on Koji agar for 2 weeks at 28°C was inoculated, test tubes were stood in a rack, and submitted to reciprocal shake culture (120 r.p.m.) at 30°C. The test tubes were then slanted at 45°. Forty-eight hours after inoculation, a solution of 2 mg of progesterone dissolved in 0.5 ml of ethanol was added and culture was continued further for 48 hours (pH 4.0–4.2). To each test tube, 10 ml of ethyl acetate was added, extracted for 3 minutes in a homogenizer, and the whole solution was filtered by suction to remove the cells. The ethyl acetate layer was transferred to a small beaker and concentrated under a reduced pressure.

Subsequently the concentrated residue was submitted to paper chromatography following the hydrazone method of Zaffaroni~4~ to examine the presence of spots other than that of progesterone. Paper chromatography conducted by the ascending technique using a water-butanol mixture should give a spot of progesterone with the Rf value of 0.13–0.17 but the products obtained from culture of microorganisms listed in Table 1 gave one spot at Rf 0.06–0.10, which agreed with the spot of 11α-hydroxyprogesterone described below.

2. Isolation and Identification of 11α-hydroxyprogesterone.

An example of experiments with Rhizopus chinensis SAITO will be given. Experiments conducted with others followed a similar procedure.

One liter of medium containing 5% glucose, 2% peptone, and 0.3% corn steep liquor was prepared and 100 ml of this medium was placed in each sterilized shaking flask of 500-ml capacity. The flasks were sterilized for 15 minutes at 15 lb., cooled (pH 5.2), and one loop of mycelium grown on Koji agar slant was inoculated. The flasks were submitted to reciprocal shake culture (120 r.p.m.) for 48 hours at 30°C. A solution of 50 mg of progesterone dissolved in 2 ml of acetone was added for each 100 ml of the medium. The total amount of progesterone used was 0.5 g. The flasks were further incubated for 48 hours (pH 4.0 at the end of culture).

The content of the flask was filtered, cells were homogenized twice with 150-ml portions of acetone, filtered, and the acetone extract was combined with the fermentation broth. The combined liquor was extracted twice with 800-ml portions of ethyl acetate, the acetate extract washed twice with 2% sodium bicarbonate solution, twice with water, and concentrated. The yield of the concentrated extract was 1.05 g.

This concentrated extract was submitted to column chromatography, using 40 g of alumina. From the portion eluted with benzene and benzene-ether (7:3), 65 mg of progesterone, m.p. 128°, was recovered. The chloroform eluate afforded 0.251 g of crystals, m.p. 154–157°. Yield, 50%. Recrystallization from ether-chloroform gave 11α-hydroxyprogesterone (I), m.p. 168.5–169.5°.


Found: C, 76.05; H, 9.22.

\[\alpha\]D +178° (c=1.4, CHCl₃).
This substance was acetylated with acetic anhydride and pyridine at room temperature and its acetate (II), m.p. 174.5-174.6°, \([\alpha]_D^{12} +127°\) (c=0.78, acetone), was obtained.

To a solution of 31 mg of this substance dissolved in 1 ml of glacial acetic acid, glacial acetic acid solution of 7 mg of chromium trioxide was added, the mixture then allowed to stand at room temperature for 2 hours, diluted with water, and extracted with chloroform. Treatment of the neutral portion afforded 21 mg of crystals which were recrystallized from methanol and confirmed to be 11-oxoprogesterone (III), m. p. 171-176°, \([\alpha]_D^{12} +220°\) (c=1, CHCl₃).

**Anal.** Calcd. for C₂₁H₃₈O₃: C, 76.79; H, 8.59.

**Found:** C, 76.60; H, 8.64.

**SUMMARY**

Oxidizability of progesterone was examined with 284 strains of *Rhizopus* sp. preserved in this Institute. Among these, 21 strains were found to have such ability. Mass culture of five of these strains was carried out and the oxidation product was identified as 11α-hydroxyprogesterone. The yield obtained was approximately 50%, using *Rh. chinensis* Saito 10-10.

**REFERENCES**


