MICROBIOLOGICAL HYDROXYLATION OF STEROIDS III. HYDROXYLATION OF PROGESTERONE BY *SYNCEPHALASTRUM* SP. (1)

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It was found, during the examination of hydroxylation ability of *Rhizopus* sp. described in a previous paper⁽¹⁾, that a strain of *Syncephalastrum* sp., preserved in this Institute, showed strong hydroxylative activity to steroids.

This fungus, when progesterone is used as the substrate, effects hydroxylation in a short period to produce dihydroxyprogesterone, m.p. 222–226°C, and no monohydroxy compound was detected. Application of the fungus for a longer fermentation period of 72 hours after the addition of progesterone effected hydroxylation to trihydroxyprogesterone, m.p. 263–265°C, although its yield was only 5% of the progesterone added. Structural studies on the product will be reported in the following paper⁽²⁾.

At the same time, some observations were made on the effect of the solvent used in the addition of progesterone, effect of the amount of substrate added, and the course of fermentation, which are described herein.

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EXPERIMENTAL

Examination of Fermentation Conditions

The method of incubation was subjected to Peterson⁽³⁾ followed as below. Shake flasks of 500 ml capacity were cotton-plugged and sterilized and 100 ml of medium, containing 5% glucose, 2% peptone, and 0.3% corn steep liquor, was placed in each flask. The flasks were then sterilized in an autoclave for 15 minutes at 15 lb and the medium showed pH 5.2. Seed from Koji agar slant was inoculated* and submitted to reciprocal shake culture (120

* When growing fungus obtained by shake culture was inoculated, large-sized pellets were formed, growth of mycelium and sugar consumption were poor, and oxygenation activity was decreased.

r.p.m.) for 48 hours in an incubator of 30°C (pH 4.0–4.2) prior to the addition of substrate.

(1) Effect of Solvents Used for the Addition of Substrate: The effect of solvents was investigated with acetone, ethanol, methanol and propylene glycol. These were also used in the study of Mann et al.⁽⁴⁾ on biogenesis of hydrocortisone from Compound S with *Cunninghamella blakesleeana*. For 100 ml of the medium, 200 mg of progesterone dissolving in 2 ml of various solvents was added and the effect of solvents on hydroxylation was examined by paper chromatography by means of the method of Zaffaroni⁽⁶⁾. The results obtained are listed in Table 1.

Table 1. Effect of Solvents Used for the Addition of Progesterone.

Solvent	pH of medium	Dry wt. of fungus (g)	Residual* progesterone	Hydroxylation product
Acetone	4.2	1.4	≡	+
Ethanol	6.0	1.7	≡	+
Methanol	6.4	1.7	±	+
Propylene glycol	5.4	1.65	±	+
No solvent	5.4	1.6	≡	+

Incubation was conducted for 72 hrs. after addition of progesterone.

* Detected by paper chromatography.

These results indicate that methanol and propylene glycol are suitable as a solvent.

(2) Effect of the Amount of Progesterone Added: Various amounts of progesterone were added to 100 ml of the medium and the products were examined by paper chromatography. Results are shown in Table 2.

It was found that the addition of 100 mg of progesterone was most effective. When 100 mg of progesterone was added as a solution in methanol,

Table 2. Effect of the Amount of Progesterone Added.

Progesterone added (mg)	Methanol	pH	Dry weight of mycelium (g)	State of mycelium	Residual progesterone		Hydroxylation product
					mycelium	broth	
50	2	6.4	2.05	pellet	—	—	+
50	4	4.4	1.05	pulp	—	—	+
100	2	6.4	1.95	pellet	—	—	+
100	4	4.4	1.01	pulp	±	—	+
150	2	6.6	1.80	pellet	+	—	+
150	4	4.6	0.95	pulp	+	—	+
200	2	6.6	2.05	pellet	+	—	+
200	4	4.8	1.00	pulp	+	+	+

oxidation was found to be affected by the volume of the solvent used, as indicated in Table 3.

Table 3. Effect of the Amount of Methanol.

Methanol (ml)	pH	State of mycelium	Dry wt. of mycelium (g)	Residual progesterone		Hydroxy- lation product
				mycelium	broth	
Nil	5.4	pellet	1.82	++	—	+
1	6.4	"	1.72	+	—	+
2	6.0	"	1.72	—	—	+
4	4.2	pulp	0.95	+	—	+
6	4.4	"	0.85	+	+	+

(3) Course of Fermentation: For 100 ml of the medium, 100 mg of progesterone dissolved in 2 ml of methanol was added, submitted to fermentation, and extraction of the reaction product was conducted every hour. The crude crystals of dihydroxyprogesterone, m.p. 206–214°C, thus obtained, was indicated as the percentage ratio of the progesterone added and shown in Fig. 1. The amount of the oxidation product reached its maximum after 6 hours. After 12 hours, oxidation progressed with the lapse of time and a trihydroxy derivative was formed.

(4) Chemical Changes during Fermentation: During the foregoing fermentation, consumption of sugar in the medium, variation of pH, and the increase of mycelium were examined. The results are graphically presented in Fig. 2.

The addition of vegetable oil (sesame oil) and silicone oil (Shin-etsu Silicone Antifoam Agent KS-66, Shin-etsu Chemical Industries, Lt., Tokyo, Japan) as an antifoam agent did not affect oxidation.

Examination of Reaction Products.

The reaction product were examined by mass culture.

(1) Oxidation products after 6 hours' incubation: Ten liters of the medium was prepared and 100 mg of progesterone dissolved in 2 ml of methanol was added for every 100 ml of the medium (total amount of progesterone used, 10 g). After fermentation for 6 hours at 30°C, the mycelium was re-

Fig. 1. Periodical Change in Formation of the Product.

moved by filtration, and the mycelium and filtrate were each extracted three times with ethyl acetate. The mycelium weighed 110 g on a dried basis. The extract solution was washed twice with 2% sodium bicarbonate solution and water, dried over anhydrous sodium sulfate, and concentrated. The concentrated extract from the mycelium was crystallized from ethanol and after the recrystallization 100 mg of ergosterol, m.p. 145–152°, was obtained. It was confirmed through both ultraviolet and infrared absorption spectra. The mother liquor remaining after removal of the crystals was evaporated and 1.65 g of the residue thus obtained was examined by paper chromatography. The majority was found to be progesterone, no oxidation product being present.

Fig. 2. Chemical Changes during Fermentation.

The crude extract (12.6 g) was obtained from the fermentation liquor, it was recrystallized from ethylacetate, and 6 g of crystals was obtained which melted at 206–214°C. The crystals were treated with chloroform, insoluble matter (0.3 g of m.p. 238–242°C) was filtered off, and the filtrate was evaporated. The residue therefrom was recrystallized twice from ethyl acetate and 2.8 g of crystals, m.p. 222–226°C, thus obtained was identified by elemental analysis as dihydroxyprogesterone.

$[\alpha]_D^{18} + 122^\circ (c=0.9, \text{CHCl}_3)$.

Anal. Calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_4$: C, 72.80; H, 8.73.

Found : C, 72.56; H, 8.65.

U.V. $\lambda_{\text{max}}^{\text{MeOH}}$: 240.2 $\text{m}\mu$ (ϵ 17000).

I.R. $\lambda_{\text{max}}^{\text{nujol}}$: 3472(OH), 1689(20-CO), 1664(3-CO), 1639(Δ^4) cm^{-1} .

Further concentration of the mother liquor afforded 1.7 g of crystals melting at 218–222°C.

A mixture of 0.45 g of this substance dissolved in 15 ml of pyridine and added with 15 ml of acetic anhydride was allowed to stand over night, poured onto ice water, and extracted with chloroform. The extract was washed consecutively with dilute hydrochloric acid, sodium bicarbonate solution, and water, dried, and the solvent was evaporated. The crude crystals (0.42 g), m.p. 185°C, were purified by column chromatography through Florisil and crystals of m.p. 188–190°C, $[\alpha]_D^{20} + 118^\circ (c=1.1, \text{CHCl}_3)$ were obtained.

Anal. Calcd. for $\text{C}_{23}\text{H}_{32}\text{O}_5$: C, 71.10; H, 8.30.

Found : C, 70.85; H, 8.27.

U.V. $\lambda_{\text{max}}^{\text{MeOH}}$: 237 $\text{m}\mu$ (ϵ 15500).

I.R. $\lambda_{\text{max}}^{\text{nujol}}$: 3690(OH), 1739(acetyl CO), 1681(20-CO), 1656(3-CO), 1621(Δ^4) cm^{-1} .

The acetate hereby obtained was found to be a monoacetate.

(2) Oxidation products after 48 hours's incubation: Fermentation was allowed to proceed for 48 hours under condition identical with those in (1); 14.75 g of the extract was obtained from the fermentation liquor. Benzene was added to the residue, the insoluble matter (1.2 g) was collected by filtration, and several recrystallizations from methanol afforded crystals of: m.p. 263–265°C (decomp.), $[\alpha]_D^{20} + 170^\circ (c=1.1, \text{MeOH})$. Yield, 0.6 g.

Anal. Calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_5$: C, 69.58; H, 8.34.

Found : C, 69.59; H, 8.44.

U.V. $\lambda_{\text{max}}^{\text{MeOH}}$: 240.2 $\text{m}\mu$ (ϵ 12700).

I.R. $\lambda_{\text{max}}^{\text{nujol}}$: 3521–3356(OH), 1695(20-CO), 1681(3-CO), 1629(Δ^4) cm^{-1} .

These data confirmed the product to be trihydroxyprogesterone.

SUMMARY

Syncephalastrum sp. was found to be capable of oxidizing progesterone to form dihydroxyprogesterone requiring a short period of incubation while trihydroxyprogesterone required a long period. These results indicated that steroids are able to be hydroxylated to trihydroxy compounds by one micro-organism. The best result in oxidation was obtained by the addition of 100 mg of progesterone to 100 ml of the medium, dissolved in 2 ml of methanol.

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