MICROBIOLOGICAL HYDROXYLATION OF STEROID IX. HYDROXYLATION OF STEROID BY *SYNCEPHALASTRUM RACEMOSUM*. (2)

TOSHINOBU ASAI, KŌ AIDA, TOKUJI TANAKA, EIJI OHKI, TAKEMASA
MATSUHISA, YOSHITO TAKEDA and TAIJI INUI

*The Institute of Applied Microbiology and Research Laboratory of
Sanraku Distillers' Co., Inc.*

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In previous studies on the oxidation of progesterone by *Rhizopus* fungus, the authors have reported that there are two kinds of *Rhizopus* species (1, 2), i.e. one that solely produces 11 α -hydroxyprogesterone and another which produces both 11 α -hydroxyprogesterone and 6 β -, 11 α -dihydroxyprogesterone. It was also reported that a strain belonging to *Syncephalastrum* produces a dihydroxyprogesterone and trihydroxyprogesterone (2, 3) both of which cannot be found in the literature. Conditions of oxidation have also been described. Concerning these progesterones, the structure of dihydroxyprogesterone was determined by TSUDA et al. (4) as 7 α , 15 β -dihydroxyprogesterone.

The present paper deals with the results obtained from investigations conducted on the taxonomical characteristics and identification of *Syncephalastrum* fungus, isolation and characteristics of trihydroxyprogesterone and oxidation of 11 α -hydroxyprogesterone, 6 β -, 11 α -dihydroxyprogesterone, compound S, hydrocortisone etc. by this strain.

EXPERIMENTAL

1. Identification of the used Strain, IAM-6801.

On the strain which was used in the previous report and described as *Syncephalastrum* sp., taxonomical studies were made. This strain was also used throughout present experiments.

(1) Diagnosis.

Appearance of cultures. Colonies on malt extract agar slant (at 30°): growing dense, at first white, becoming gray with sporangia on the upper part of slant; on koji extract agar slant (at 30°): turf somewhat dense, covered by small sporangia, becoming dark gray; on Pfeffer-oryzanin agar slant (5) (at 30°): turf somewhat dense or sparse, covered by sporangia, becoming light brownish gray. Sporangioophores simple at first, later becoming branched, with short straight or curved branches racemosely arranged, apically capitate to form vesicles, usually septate, globose, wall rough, 38 μ ×29 μ (11–57 μ ×11–56 μ) in diameter. Vesicles covered with abundant projections bearing sporangia without basal cells, globose, ellip-

tical or irregular in shape, $66\mu \times 59\mu$ ($23\text{--}120\mu \times 23\text{--}110\mu$) in diameter. Sporangia (as termed by ZYCHA (6) "part-sporangia") at first tubular cells, later septate, containing 3-spores in a series, readily breakable to spores

Fig. 1. Photographs of *Syncephalastrum racemosum*

in old cultures. Spores globose, elliptical, ovoid, polygonal, rarely cocoon- or kidney shaped, or irregular in shape, $4.6\mu \times 4.2\mu$ ($2\text{--}8\mu \times 2\text{--}6\mu$) in diameter. Zygosporangia and chlamydospores, not observed.

Gas formation from several carbohydrates. On Pfeffer solution containing 5% glucose, fructose, galactose, sucrose, maltose or starch respectively (at 30°), wet mycelial mat formed and gas not formed from them (in fermentation test-tubes).

(2) Identification.

It is obvious that this strain belongs to the order Mucorales according to the fact that its hyphae are non-septate and asexual spores occur in sporangia and are non-motile. Then because of the formation and shape of part-sporangia, it belongs to the sub-order *Cephalidaceae* (by ZYCHA (6) or *Piptocephalidaceae* (by BESSEY (7)), furthermore to the genus *Syncephalastrum* for the absence of basal cells.

The genus *Syncephalastrum* was classified by ZYCHA (6) in regard to the shape and dimensions of spores, as follows: (i) spores globose, $2.5\text{--}5\mu$ (*S. racemosum*) and (ii) spores ovoid, $5\text{--}8\mu$ (*S. fuliginosum* and *S. javanicum*). According to this key, this strain rejects the latter and almost accords with ZYCHA's (6) description of *S. racemosum* in respect to the dimensions of several organs and the number of spores contained in sporangia, except

that this strain has not always septa under vesicles. Whereas, the present strain distinctly differs from *S. nigricans*, a synonym of *S. racemosum*, which has only 2-3 spores in sporangia.

Therefore, this strain is decided as *Syncephalastrum racemosum* (Cohn) SCHROETER (8).

2. Oxidation of Progesterone.

In order to isolate the oxidation products, tank culture was performed with a 50 l. stainless fermentation tank. To a medium of 30 l. with a composition of 5% glucose, 2% peptone, 0.3% corn steep liquor, 3 l. of culture precultivated for 48 hours on a medium of the same composition in a shaking flask was inoculated. After cultivation at 30° for 24 hours, 30 g.

Fig. 2. Hydroxylation of steroids by *Syncephalastrum racemosum* IAM-6801

(I) progesterone	(II) 7 α ,15 β -dihydroxyprogesterone
(III) 7 α ,14 α ,15 β -trihydroxyprogesterone	(IV) 11 α -hydroxyprogesterone
(V) 6 β , 11 α -dihydroxyprogesterone	(VI) Reichstein's compound S
(VII) 11-epihydrocortisone.	

of progesterone dissolved in an amount of 600 ml. of methanol was added and fermentation was continued for a further 48 hours (aeration, 30 l. per min. (eq. volume), stirring, 250–300 r.p.m.). A comparison of the fermentation course with shake culture in a 500 ml. flask is given in Fig. 4. From the figure it was evident that tank fermentation proceeds more rapidly. The entire fermentation course required 72 hours and consumption of sugar in the medium was 93%, being quite satisfactory. After fermentation was completed the culture broth was separated into the mycelium and the filtrate. The mycelium was extracted with acetone, the extract solution and filtrate collected and extracted three times with an equal volume of ethyl acetate. Then the extract solution was evaporated to approx. 2 l. and after washing with 2% sodium bicarbonate solution and distilled water, drying over anhydrous sodium sulfate, the solvent was distilled under reduced pressure, and a residue, 16.5 g., was obtained. Washing with chloroform resulted 5 g. of an insoluble matter. Further treatment with chloroform and consecutive recrystallization from methanol gave needle crystals, 2.3 g, m.p., 262–65° (decomp.) Yield, approx. 7.7%.

$$[\alpha]_D^{25} + 103^\circ \quad (c=0.5, \text{MeOH}).$$

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

Found : C, 65.59, H, 8.44

U. V. $\lambda_{\text{max}}^{\text{MeOH}}$: 240.2 m μ (ϵ 12,700)

I. R. $\lambda_{\text{max}}^{\text{Nujol}}$: 3,356–3,521 (OH), 1,695 (20–CO), 1,681 (3–CO), 1,629 (Δ^4) cm^{-1}

From the above data, the product is considered to be identical with progesterone as it had three hydroxy groups. Studies on the position of the hydroxy groups conducted by TSUDA, OHKI et al (9) gave evidence that this product is 7 α , 14 α , 15 β -trihydroxyprogesterone. A sample of 0.32 g. was dissolved in 2 ml. of pyridine, added 2 ml. of acetic anhydride and the mixture was allowed to stand overnight. Then, 3 ml. of methanol was further added and it was left for a while. Distillation of the solvent under reduced pressure afforded an oily residue, which upon treatment with ether and recrystallization from acetone afforded crystals, m.p. 228–30°.

$$[\alpha]_D^{25} + 12.6^\circ \quad (c=0.62, \text{MeOH})$$

Anal. Calcd. for $\text{C}_{23}\text{H}_{32}\text{O}_6$: C, 68.29, H, 7.97

Found : C, 68.15, H, 7.97

U. V. $\lambda_{\text{max}}^{\text{MeOH}}$: 235.7 (ϵ 15,000)

I. R. $\lambda_{\text{max}}^{\text{Nujol}}$: 3,356–3,584 (OH), 1,736 (Ac) 1,692 (20–CO), 1,650 (3–CO), 1,616 (Δ^4) cm^{-1} ,

From the above data, the product was confirmed to be a mono-acetate of trihydroxyprogesterone. The chloroform soluble fraction was distilled off and the residue recrystallized from ethyl acetate gave crystals, 5.8 g, m.p.,

225°–226°, identical with 7 α ,15 β -dihydroxyprogesterone previously reported (3). Yield, 19.3%.

3. Oxidation of 11 α -progesterone.

After 48 hours' cultivation in the medium described above, 100 mg of 11 α -hydroxyprogesterone per 100 ml. medium dissolved in 2 ml. of methanol was added and microbiological oxidation was continued. Consumption of sugar in the medium, mycelial growth and pH variation almost agreed with data given in Fig. 2 of Part (VI) (3). In this case changes occurring during fermentation were observed to be identical with the case of progesterone used as the substrate. Paper-chromatograms of oxidation products formed after both 6 and 48 hours' fermentation are given in Fig. 4.

Fig. 3. —○—○— residual sugar (tank culture)
 —○—○— pH (")
 ---△---△--- residual sugar (shaking culture)
 ---△---△--- pH (")

(1) *Oxidation products formed after 6 hours' incubation.* Two hundred ml. of the medium was extracted with ethyl acetate, evaporated under reduced pressure and two products were obtained from difference of solubility. About 20 mg. of the product having lower solubility was obtained and repeated recrystallizations from ethyl acetate afforded crystals.

Yield, approx. 10%, m.p. 245–7°, mixed melting point with a known 6 β , 11 α -dihydroxyprogesterone (m.p. 245–48°) did not depress.

$[\alpha]_D^{20} +152^\circ$ ($c=0.97$, CHCl_3),

I. R. $\lambda_{\text{max}}^{\text{NaOH}}$: 3,448 (OH), 1,698 (20-CO), 1,661 (3-CO), 1,618 (Δ^4) cm^{-1}

All these data confirmed the product to be identical with 6 β , 11 α -dihydroxyprogesterone. The crystals obtained from the portion readily soluble in ethyl acetate were recrystallized from methanol, m.p. 164°, and confirmed to be identical with 11 α -hydroxyprogesterone.

(2) *Oxidation products obtained after 48 hours' incubation.* A culture liquor of 200 ml. was extracted with ethyl acetate and treated in the same manner as above. In this case, however, only 6 β , 11 α -dihydroxyprogesterone was obtained. Only a single spot could be detected even when paper chromatography was applied. Yield, 180 mg (approx. 90%). No substance with three hydroxy groups was produced.

4. *Oxidation of 6 β , 11 α -dihydroxyprogesterone.*

This strain is capable deriving the hydroxygroup into three positions of the structure of progesterone, used as the substrate. For this reason, oxidation by the addition of 6 β , 11 α -dihydroxyprogesterone at growth

Fig. 4. Oxidation products of 11 α -hydroxyprogesterone

Solvent: water saturated butanol

Spraying reagent: potassium
iodoplatinate

Developed for 16 hrs. by Zaffaroni's
ascending method.

period under highly activate oxidation was attempted. The seed was cultivated for 48 hours after inoculation and 100 mg. of 6 β , 11 α -dihydroxyprogesterone (m.p. 205–208°) per 100 ml.-medium dissolved in 2 ml. of methanol (slightly insoluble) was added, continued to following fermentation for 6 hours and 48 hours, and extrated with ethyl acetate respectively. Although isolation of oxidation products was attempted, only 6 β , 11 α -dihydroxyprogesterone was obtained at each stage. Again in this case, only a single spot could be detected by paperchromatography. From the above results it may be said that when 11 α -hydroxyprogesterone is employed as the substrate, this strain oxidizes only position 6, while other positions are not affected.

5. Oxidation of Reichstein's Compound S.

The authors next attempted the oxidation of Reichstein's compound S (11-desoxy,17-hydroxycorticosterone) which has two hydroxy groups. The procedure was as follows: the strain was cultivated for 48 hours in the medium mentioned above; 100mg of Reichstein's compound S per 100ml. culture medium dissolved in 2 ml. of methanol was then added and microbiological oxidation was continued. At periods of 6 and 48 hours after addition of the substrate, the medium was extracted with ethyl acetate respectively, and the products examined. Paperchromatograms of the oxidation products after 48 hours' incubation are shown in Fig. 5.

(1) Oxidation products formed after 6 hours' incubation.

The extract solution was evaporated and the products examined by paper-chromatography but no spot expect that of Reichstein's compound S, the substrate could be detected. From the extract solution, crystals, m.p., 198° were obtained. This crystals did not show depression of melting point on mix melting with Reichstein's compound S.

(2) Oxidation products formed after 48 hours' incubation.

Evaporation of the extract solution gave a crystal, m.p. 208-210°. This crystal evidently differed from compound S. For such reason mass culture was performed in order to isolate and identify this product.

(3) Identification of oxidation products.

One hundred ml. of each of the above medium of 10 l. was poured into shaking flasks and submitted to mass culture under identical conditions as those described above. Cultivation was performed 48 hours after inoculation of the seed, 100 mg. of Reichstein's compound S per 100 ml. medium dissolved in 2 ml. of methanol was added and fermentation continued for a further 48 hours. The consumption of Reichstein's compound S in the medium was 10 g. in total. After cultivation was finished, broth was extracted three times with, ethyl acetate. The ethyl acetate attract was washed with 2% sodium bicarbonate solution and aq. destilla, dried over anhydrous sodium sulfate and evaporated under reduced pressure. The concentrates were treated with chloroform, and the solvent was evaporated

Fig. 5. Oxidation products of Reichstein's Compound S.

Solvent: toluen: dioxan 4:1 (saturated with propylen glycol). Tōyō Filter paper treated with a mixture of methanol and propylen glycol (4:6).

Spraying agent: TPTZ Developed for 10 hrs by Zaffaroni's descending method

under a reduced pressure. The insoluble portion gave 4 g. of solid matter. Recrystallization from acetone afforded the crystals (I), 2.8 g., m.p., 208–210°.

$$[\alpha]_D^{16} + 107^\circ \text{ (c=0.73, CHCl}_3\text{)}$$

Anal. Calcd. for $C_{21}H_{30}O_5$: C, 69.58, H, 8.35

Found : C, 69.45, H, 8.30

U. V. $\lambda_{\text{max}}^{\text{MeOH}}$: 241.4 m μ (ϵ 17,800)

I. R. $\lambda_{\text{max}}^{\text{Nujol}}$: 3,497 (OH), 1,706 (20–CO), 1,647 (3–CO), 1,613 (Δ^4) cm^{-1}

The infrared spectrum is given in Fig. 6.

From the above data, the product is identical with 11-epihydrocortisone. In order to confirm this result the authors proceeded to prepare derivatives. A quantity of 0.3 g of the crystals (I) was dissolved in pyridine, added with acetic anhydride and after being allowed to stand overnight, poured onto ice water, extracted with chloroform, recrystallized from a mixture of chloroform-ether. Thus, the crystals (II) an acetylated compound, m.p., 200–202° was obtained.

$$[\alpha]_D^{15} + 120^\circ \text{ (c=1, CHCl}_3\text{)}$$

Anal. Calcd. for $C_{25}H_{34}O_7$: C, 67.24, H, 7.64

Found : C, 67.06, H, 7.74

The above data confirmed this crystal (II) to be a diacetate of 11-epihydrocortisone.

A portion of 0.2 g. of the crystal (II) was successively dissolved in a solution of water–methanol (1 : 2) in which 2 g of acidic potassium carbonate was dissolved. After being allowed to stand for two days the solution was neutralized with dilute hydrochloric acid, diluted with water, extracted with ethyl acetate, washed with a sodium bicarbonate solution, dried over anhydrous sodium sulfate and the solvent was distilled and recrystallized from a mixture of chloroform-ether. Crystals (III), m.p., 203–5° were obtained.

Anal. Calcd. for $C_{23}H_{32}O_6$: C, 68.32 H, 7.92

Found : C, 68.32 H, 7.92

These data confirmed the product to be a monoacetate of 11-epihydrocortisone.

A sample of 0.18 g. of the crystals (I) was dissolved in 10 ml. of glacial acetic acid, added 5 ml. of a solution of 0.43 g. anhydrous chromic acid dissolved in 10% acetic acid, allowed to stand overnight at room temperature and then diluted. After the neutral fraction was extracted with ether, washed with sodium bicarbonate solution and dried over anhydrous sodium sulfate, the solvent was distilled. A crude crystal was then obtained. purification by alumina column chromatography and recrystallization from a mixture of chloroform-ether gave crystals, 75 g. m.p., 210–212°.

Anal. Calcd. for $C_{19}H_{24}O_3$: C, 75.97, H, 8.05

Found : C, 75.68, H, 8.07

By mix melting point of this crystal with adrenosterone obtained from chromic acid oxidation of cortisone, and comparison of infrared spectrum this crystal was identified to be adrenosterone. Thus, the crystal (1) was confirmed to be 11-epihydrocortisone.

6. Oxidation of hydrocortisone.

Hydrocortisone was added as the substrate and the broth was extracted with ethyl acetate at periods of 6 and 48 hours after addition of the substrate. No oxidation products except the substrate could be found.

Fig. 6. Infrared spectrum of 11-epihydrocortisone [I] and Reichstein's compound S [II]

DISCUSSION

From the above experiments conducted with *Syncephalastrum racemosum* I. A. M. 6801, it is clear that the hydroxy group is induced at five positions of the steroid ring, namely, 6β , 7α , 11α , 14α , and 15β . However, in each case, the progress of reaction is dependent on the kind of steroid employed.

This may be elucidated from the fact that progesterone which does not possess a hydroxy group is most readily oxidized—the first product formed being a 7α , 15β -dihydroxyl compound and, as oxidation proceeds a 7α , 14α , 15β -trihydroxyl compound is formed. However, it is worthy to note that even in the same progesterone, reaction differs according to the position of the hydroxyl group. As for example, when a certain progesterone with a hydroxy group attached at the 11α -position so used as the substrate, an entirely different reaction occurs, offering a 6β -hydroxyl compound. Moreover, the

reaction is much more retarded than in the case of progesterone. As for Reichstein's compound S, which still has one more hydroxyl group, oxidation products are not observed in a short period, the production of 11-epihydrocortisone is first recognized only after a long cultivation period. Furthermore, when 6 β ,11 α -dihydroxyprogesterone and hydrocortisone were used as the substrates no any oxidation products were formed.

As it is shown here, a considerable difference in oxidation is due to a slight change in the structure of the steroid substrate. This is certainly a markable characteristic of microbiological oxidation which is also observed in the oxidation of progesterone and 11-desoxycorticosterone (10) by *Rhizopus arrhizus*, and that of progesterone and 11 α -hydroxyprogesterone (11) by *Calenectria decora*.

SUMMARY

On the strain which was used in the previous report (2, 3) and described as *Syncephalastrum* sp., taxonomical studies were made and it was decided as *Syncephalastrum racemosum* (Cohn) Schroeter.

This fungus formed 7 α ,15 β -dihydroxyprogesterone from progesterone in a short period of incubation, while 7 α ,14 α ,15 β -trihydroxyprogesterone in a long period. By this strain, 6 β ,11 α -dihydroxyprogesterone and 11-epihydrocortisone were also formed from 11 α -hydroxyprogesterone and compound S, respectively. However, no oxidation products could be found from 6 β ,11 α -dihydroxyprogesterone and hydrocortisone.

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