UDC 547.92 : 581.13 : 582.12

71. Kyosuke Tsuda, Toshinobu Asai, Eiji Ohki, Atsuji Tanaka, and Masako Hattori : Microbiological Hydroxylation of Steroids. IV. 7α,15β-Dihydroxylation of Progesterone by *Syncephalastrum* sp. (2).

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In recent years, a new synthetic process has been found[1] to introduce a substituent into steroid skeleton by enzymatic hydroxylation of microörganisms for the preparation of adrenocortical hormones whose chemical synthesis would be extremely difficult and require a long process.

As was reported in the preceding paper,[2,3] the authors compared oxygenation ability of *Rhizopus* and *Aspergillus* sp. kept in this institute and selected some strains having ability of introducing a hydroxyl group into the 11α-position of progesterone and 17α, 21-dihydroxy-4-pregnene-3,20-dione (Reichstein's Compound S). It was thereby found, during the selection, that a strain of *Syncephalastrum* sp. kept in this institute had a strong action to hydroxylate steroids.[4]

This fungus, when Reichstein's Compound S and 11α-hydroxyprogesterone are used as the substrate, effects hydroxylation to produce 11-epihydrocortisone and 6β,11α-dihydroxyprogesterone, respectively, and is similar to *Rhizopus* sp. in the mode of hydroxylation.

When progesterone is used as a substrate, however, in a short-period fermentation, it was transformed to dihydroxyprogesterone (I), m.p. 222~226°, $[\alpha]_D^{18}$ +122°, and monohydroxy compound was not detected. Application of the fungus for a longer fermentation period after the addition of progesterone effected hydroxylation to trihydroxyprogesterone, m.p. 263~265°, $[\alpha]_D^{20}$ +170°.[4]

The dihydroxyprogesterone so obtained was found by later studies to have the hydroxyls in the 7α- and 15β-positions. Such 7α,15β-dihydroxylation is unknown in literature, although the introduction of one hydroxyl group in the 7-[5,6] or 15-position[1a,6~8] has been found in some microörganisms.

As the ultraviolet absorption of (I) did not shift from that of progesterone[9] and ferric chloride reaction[10] and Porter-Silber[11] test are negative, hydroxylation of 2-, 6β-, 16-, and 17-positions can be excluded. Acetylation of (I) with acetic anhydride and pyridine affords a monoacetate (II), m.p. 188~190°, and the other hydroxyl group is not

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1) (a) J. Fried, R.W. Thoma, D. Perlman, J.E. Herz, A. Borman : Recent Progr. in Hormone Research, **11**, 149(1955); (b) S.H. Eppstein, P.D. Meister, H.C. Murray, D.H. Peterson : Vitamins and Hormones, **14**, 359(1956); A. Wettstein : Experientia, **11**, 465(1955); E. Vischer, A. Wettstein : Angew. Chem., **69**, 456(1957).
2) T. Asai, K. Tsuda, K. Aida, E. Ohki, T. Tanaka, M. Hattori : J. Gen. and Appl. Microbiology, **4**, 63(1958).
3) H. Iizuka, A. Naito, M. Hattori : *Ibid.*, **4**, 67(1958).
4) T. Asai, K. Aida, E. Ohki, T. Tanaka, M. Hattori : *Ibid.*, **4**, 79(1958); Nippon Nôgei-kagaku Kaishi, **32** (1958), in press.
5) A. Wettstein, E. Vischer : Experientia, **8**, 423(1952).
6) Ch. Meystre, E. Vischer, A. Wettstein : Helv. Chim. Acta, **38**, 381(1955).
7) S. Bernstein, J.F. Feldman, *et al.* : Chem. & Ind. (London), **1956**, 111.
8) B. Klüger, S. Siebert, A. Schubert : Naturwiss., **2**, 40(1957).
9) C.W. Bird, R.C. Cookson : J. Chem. Soc., **1956**, 3675.
10) L.F. Fieser, M.A. Romero : J. Am. Chem. Soc., **75**, 4716(1953).
11) R.H. Silber, C.C. Porter : J. Biol. Chem., **210**, 923(1954).

acetylated. From analysis of its infrared absorption[12] the hydroxylation of 21-position is excluded.

Saponification of the monoacetate (II) with potassium hydroxide or mildly with potassium hydrogen carbonate results in dehydration of the acetylated hydroxyl group, without recovery of the starting material, and a dienone (III), m.p. 217~218°, is obtained. Its ultraviolet absorption has shifted to a longer wave-length region of 285 mμ. It is linear 4,6-dien-3-one corresponding to 6,7-dehydrocorticosterone (283 mμ) and 4,6,23-ergostatrien-3-one (280 mμ). Its infrared spectrum exhibits a new absorption at 1590~1587 cm^{-1} of strong intensity. The formation of (III) has proved the presence of a hydroxyl at 7-position, the β-position of the conjugated ketone system, in the acetate (II). (III) is also obtained on boiling (I) with potassium hydroxide in methanol.

Oxidation of (III) with chromium trioxide and pyridine affords a ketone (V), m.p. 192~194°, which is also obtained on oxidation of the monoacetate (II) with chromium trioxide in acetic acid, followed by saponification of the keto-acetate (IV), m.p. 184~186°, so obtained, with potassium hydrogen carbonate. The ketone compound (V) here obtained exhibited infrared absorptions at 1730 cm^{-1} (Nujol) and 1733 cm^{-1} ($CHCl_3$) corresponding to a five-membered cyclic ketone[12] and was considered to have the carbonyl at 15- or 16-position. However, its ultraviolet absorption was at 284.8 mμ and there was no absorptions in the shorter wave-length region, that (V) could not be a β-diketone. Since the hydroxyl in (I) that is not acetylated is stable to alkalis, substitution in 16-position is out of the question.[13] Therefore, one of the hydroxyl must be in 15-position and (I) is consequently assumed as 7,15-dihydroxyprogesterone.

As the hydroxyl group in 15-position is not acetylated, its configuration is considered to be 15β. Partial hydrogenation of (III) with palladium-on-charcoal in alkaline medium afforded known 15β-hydroxyprogesterone (VII), m.p. 199~202°, which had been obtained by Fried, *et al.*[1a] by oxygenation of progesterone with *Phycomyces* sp. By comparing the difference of molecular rotations between (I) (M_D +423) and (VII) (M_D +522), the hydroxyl group of 7-position may be assigned 7α-configuration[6] by its levorotatory contribution.

The 15-ketone compound (V) is unstable in alkali and to adsorbents like alumina and Florisil, coloring reddish. When left in ethanol-hydrochloric acid mixture, it underwent isomerization to (VI), m.p. 164~165°. Its ultraviolet absorption shifted to a slightly longer wave-length region than that of (V) and its infrared absorption did not agree in the finger print region.

Originally, *cis*-configuration is stable in hydroindanone series but it has been proved that in C/D ring of 15-oxosterols the *trans* ring-juncture is more stable.[14] In 15-ketone compounds of spirostane[15~17] and 20-oxopregnane[15] series, the C/D ring juncture undergoes isomerization from *trans* to *cis*.

Following the report of Fried, *et al.*,[1a] oxidation of (VII) with chromium trioxide and pyridine afforded 15-oxoprogesterone (VIII), m.p. 151~155°, which underwent isomerization to 14-*iso* compound (IX), m.p. 208~210°, in the presence of hydrogen ion. (IX) is also obtained by partial hydrogenation of (VI) with palladium-charcoal in alkaline medium. From such a fact, it is assumed that (V), obtained as above, had undergone ring conversion to (VI), as shown in Chart 1.

12) R.N. Jones, *et al.*: J. Am. Chem. Soc., **70**, 2025(1948).
13) R.E. Marker, D.L. Turner, R.B. Wagner, P.R. Ulschfter: *Ibid.*, **63**, 779(1941).
14) D.H.R. Barton: Chem. & Ind. (London), **1953**, 616.
15) C. Djerassi, L.B. Higl, J. Fried: J. Am. Chem. Soc., **77**, 3673(1955).
16) D.L. Klass, M. Fieser, L.F. Fieser: *Ibid.*, **77**, 3829(1955).
17) C. Djerassi, G.H. Thomas, *et al.*: *Ibid.*, **79**, 3835(1957).

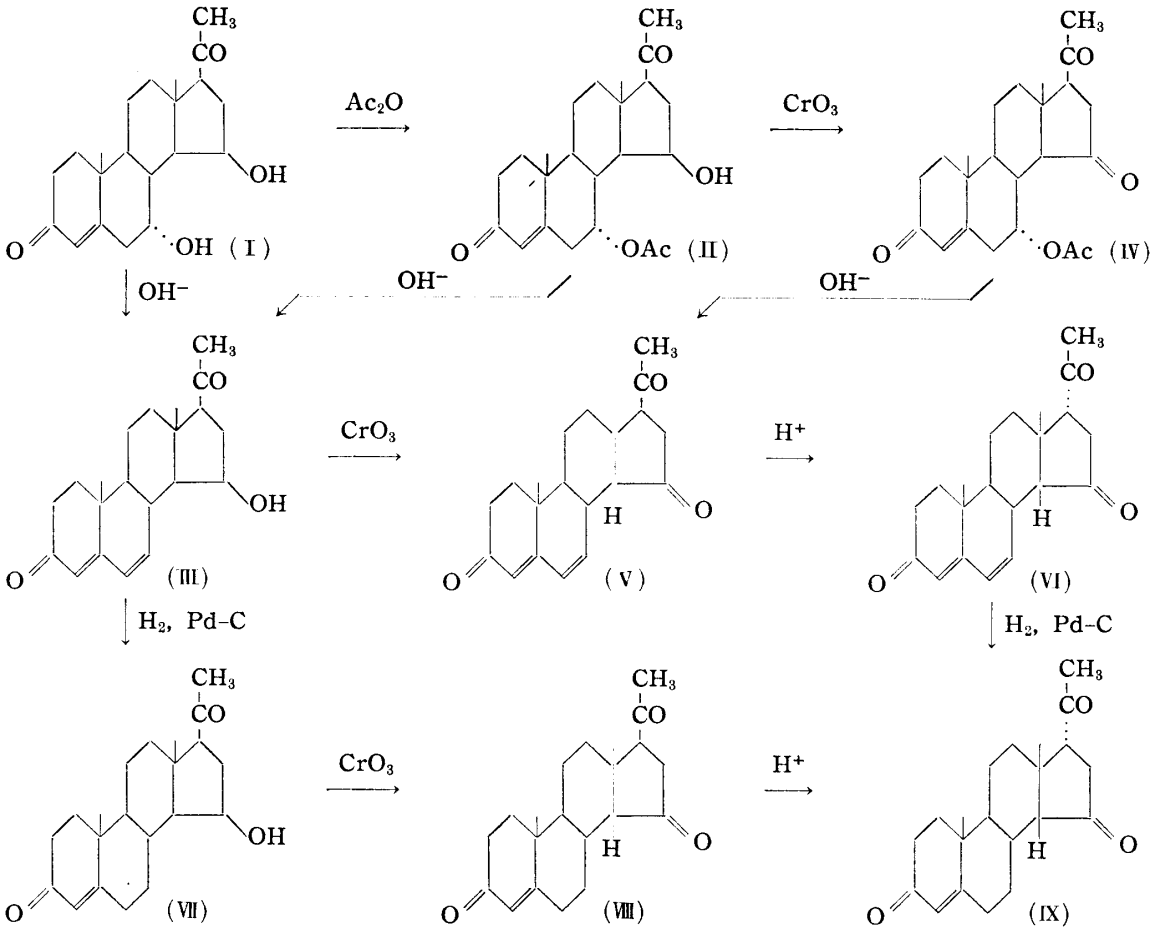

Chart 1.

The authors are indebted to Miss H. Yamanouchi and Mr. Y. Sato of this Institute for elemental microanalyses and to Mr. K. Aizawa of the Agricultural Chemical Department of this University for infrared spectral measurements. Part of the expenses for the present work was defrayed by a Grant in Aid for Scientific Research and General Research Fund for 1957, both provided by the Ministry of Education, which are herein gratefully acknowledged.

Experimental

(All m.p.'s are uncorrected)

7α,15β-Dihydroxyprogesterone (7α,15β-Dihydroxy-4-pregnene-3,20-dione)(I)—Shake flasks of 500-cc. capacity were cotton-plugged and sterilized, and 100 cc. 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 mins. at 15 lbs. and the medium showed pH 5.2. Seeding from Koji-agar slant was inoculated and submitted to reciprocal shake culture (120 r.p.m.) for 48 hrs. in an incubator of 30° (pH 4.0~4.2) and 100 mg. of progesterone dissolved in 2 cc. of MeOH was added for every 100 cc. of the medium (total amount of progesterone used, 10 g.). After incubation for 6 hrs. at 30° the mycelium was collected by filtration, and mycelium and filtrate were each extracted 3 times with AcOEt. The extract solution was washed twice with 2% $NaHCO_3$ solution and evaporated.

The concentrated extract (12.69 g.) precipitated crystals on standing and filtration afforded 6 g. of crystals melting at 206~214°. The crystals were treated with $CHCl_3$, insoluble matter (0.3 g. of m.p. 238~242°) was filtered off, and the filtrate was evaporated. The residue therefrom was recrystallized twice from AcOEt and 2.8 g. of crystals (m.p. 222~226°) so obtained was identified by elemental analysis as dihydroxyprogesterone (I). $[\alpha]_D^{18}$ +122°(c=0.9, $CHCl_3$). *Anal.* Calcd. for $C_{12}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.56; H, 8.65. U.V. λ_{max}^{MeOH} 240.2 mμ(ε 17000). I.R. (in Nujol) cm^{-1}: 3472(OH), 1689(20-CO), 1664(3-CO), 1639(Δ^4).

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

7α,15β-Dihydroxy-4-pregnene-3,20-dione 7-Monoacetate (II)—A mixture of 0.45 g. of this substance dissolved in 15 cc. of pyridine and added with 15 cc. of Ac_2O was allowed to stand over night, poured into ice water, and extracted with $CHCl_3$. The extract was washed consecutively with dil. HCl,

$NaHCO_3$ solution, and water, dried, and the solvent was evaporated. The crude crystals (0.42 g.), m.p. 185°, were purified by column chromatography through Florisil and crystals of m.p. 188~190°, $[\alpha]_D^{20}$ +118°(c=1.1, $CHCl_3$), were obtained. *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found : C, 70.85; H, 8.27. U.V. λ_{max}^{MeOH} 237 mμ(ε 15500); I.R. cm^{-1} (in Nujol) : 3690(OH), 1739(acetyl CO), 1681(20-CO), 1656(3-CO), 1621(Δ^4).

15β-Hydroxy-4,6-pregnadiene-3,20-dione (III)—(i) A mixture of 0.5 g. of (I) in 60 cc. of 0.5% MeOH containing 1 g. of KOH was boiled for 1 hr., cooled, diluted with water, and extracted 3 times with $CHCl_3$. The extract was washed with water, dried over anhyd. Na_2SO_4, and $CHCl_3$ was evaporated. The residue was purified through chromatography with 20 g. of Florisil and 0.4 g. of residue obtained by elusion with $CHCl_3$-acetone mixture (1:9 and 2:8) was recrystallized from AcOEt to 0.24 g. of 15β-hydroxy-4,6-pregnadiene-3,20-dione (III), m.p. 216~218°, $[\alpha]_D^{16}$ +159°(c=0.9, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found : C, 76.57; H, 8.53. U.V. λ_{max}^{MeOH} 284.8 mμ(ε 31000); I.R. cm^{-1} (in Nujol) : 3546(OH), 1706(20-CO), 1664(3-CO), 1616, 1587(4,6-diene); (in $CHCl_3$) : 3509(OH), 1698(20-CO), 1650(3-CO), 1618, 1590(4,6-diene) (0.1-mm. cell).

(ii) A mixture of 0.1155 g. of (II) in 15 cc. of MeOH containing 0.127 g. of KOH was boiled for 1 hr., treated as above, and 0.1033 g. of product was obtained. It was recrystallized from AcOEt to crystals of m.p. 218~220°, $[\alpha]_D^{16}$ +157°(c=1, $CHCl_3$). Its infrared spectrum was identical with that of (III) obtained by the method (i).

The same substance (III) was obtained by the addition of 0.5 g. of $KHCO_3$ to MeOH solution of 0.146 g. of (II), water and MeOH added to produce a homogeneous solution, allowed to stand for 2 days, and treated as in the foregoing examples.

7α-Hydroxy-4-pregnene-3,15,20-trione 7-Monoacetate (IV)—To a solution of 0.1266 g. of (II) dissolved in 5 cc. of AcOH a solution of 10% AcOH containing 64 mg. of CrO_3 was added dropwise under cooling with ice and the mixture was allowed to stand for 2 hrs. at room temperature. This was diluted with water, extracted with $CHCl_3$, and the extract was washed consecutively with water, $NaHCO_3$ solution, and water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.1158 g. of crystals, melting at 165~172°, was obtained. Two recrystallizations from $CHCl_3$-ether mixture afforded (IV), m.p. 184~186°, $[\alpha]_D^{16}$ +68.7°(c=1.6, $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.15; H, 7.79. U.V. λ_{max}^{MeOH} 237 mμ(ε 16370). I.R. cm^{-1}(in $CHCl_3$) : 1736(acetyl CO), 1712(15-CO), 1695(20-CO), 1661(3-CO), 1616(4-ene) (0.1-mm. cell).

4,6-Pregnadiene-3,15,20-trione (V)—(i) A solution of 0.154 g. of (III) dissolved in 2 cc. of pyridine was added to a cold solution of 4 cc. of pyridine containing 400 mg. of CrO_3 and the mixture was allowed to stand for 2 days. The mixture was diluted with $CHCl_3$, insoluble matter was filtered off, and the $CHCl_3$ solution was washed consecutively with water, dil. HCl, $NaHCO_3$ solution, and water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.1166 g. of the residue hereby obtained was recrystallized twice from AcOEt to 4,6-pregnadiene-3,15,20-trione, m.p. 192~194°, $[\alpha]_D^{16}$ +161° (c=1.34, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{26}O_3$: C, 77.27; H, 8.03. Found : C, 77.03; H, 8.03. U.V. λ_{max}^{MeOH} 283.2 mμ(ε=27600). I.R. cm^{-1}(in Nujol) : 1730(15-CO), 1698(20-CO), 1669(3-CO), 1616, 1587(4,6-diene); (in $CHCl_3$) : 1733(15-CO), 1701(20-CO), 1653(3-CO), 1618, 1592(4,6-diene) (0.1-mm. cell).

(ii) A solution of 0.1 g. of (III) dissolved in MeOH was added to hydr. MeOH solution containing 0.5 g. of $KHCO_3$ and the solution was allowed to stand for 2 days. This was diluted with water, extracted with $CHCl_3$, and the extract was dried over anhyd. Na_2SO_4. Evaporation of the solvent left 0.1162 g. of residue which was recrystallized from ether to 72 mg. of crystals melting at 178~185°, alone and in admixture with (V) obtained by the method (i). The infrared and ultraviolet spectra were also in good agreement. *Anal.* Calcd. for $C_{21}H_{26}O_3$: C, 77.27; H, 8.03. Found : C, 76.53; H, 8.09.

Isomerization of 4,6-Pregnadiene-3,15,20-trione to (VI) by Acid—One drop of HCl was added to a solution of 0.1423 g. of (V) dissolved in 30 cc. of MeOH and the mixture was allowed to stand over night, during which the solution acquired a faint yellowish tint. The solution was diluted with water, extracted three times with $CHCl_3$, and the extract was washed with $NaHCO_3$ solution and water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.1514 g. of the residue so obtained was recrystallized from benzine-ether mixture to 81.1 mg. of crystals melting at 164~165°, $[\alpha]_D^{16}$ +212°(c=0.87, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{26}O_3$: C, 77.27; H, 8.03. Found : C, 76.93; H, 7.96. U.V. λ_{max}^{MeOH} 286.7 mμ(ε 24400). I.R. cm^{-1}(Nujol): 1733(15-CO), 1698(20-CO), 1661(3-CO), 1618, 1590(4,6-diene).

15α-Hydroxy-4-pregnene-3,20-dione (VII)—A solution of 0.3244 g. of (III) dissolved in 40 cc. of MeOH containing 0.03 g. of KOH was catalytically reduced with 1% Pd-C (0.14 g.). After absorbing about 2.2 cc. of H_2 the hydrogenation was stopped. After removal of the catalyst, the solvent was diluted with H_2O, extracted with $CHCl_3$, and the extract was washed with water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.2565 g. of the residue so obtained was purified by alumina chromatography and recrystallized to 15β-hydroxy-4-pregnene-3,20-dione (VII), m.p. 199~202°; $[\alpha]_D^{9}$ +158° (c=1.2, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found : C, 76.53; H, 9.17. U.V. λ_{max}^{MeOH} 241.4 mμ(ε 18600). I.R. cm^{-1} (in Nujol) : 3534(OH), 1695(20-CO), 1664(3-CO), 1613(Δ^4).

4-Pregnene-3,15,20-trione (VIII)—A solution of 0.120 g. of (VII) in 2 cc. of pyridine was added to a solution of 4 cc. of pyridine containing 500 mg. of CrO_3, and the mixture was allowed to stand for 2 days. The mixture was diluted with $CHCl_3$, insoluble matter was filtered off, and the $CHCl_3$ solution was washed consecutively with water, dil. HCl, $NaHCO_3$ solution, and water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.080 g. of the residue hereby obtained was recrystallized from ether to 4-pregnene-3,15,20-trione, m.p. 151~155°; $[\alpha]_D^{10}$ +192°(c=0.5, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found : C, 76.40; H, 8.60. U.V. λ_{max}^{MeOH} 240 mμ(ε 19500). I.R. cm^{-1} (in Nujol) : 1727(15-CO), 1695(20-CO), 1663(3-CO), 1623(Δ^4).

14-Iso Compound (IX) of (VIII)—(i) One drop of HCl was added to a solution of 0.122 g. of (VIII) dissolved in 30 cc. of MeOH and the mixture was allowed to stand over night. The solution was diluted with water, extracted with $CHCl_3$, and the extract was washed with $NaHCO_3$ solution and water. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off and 0.160 g. of the residue so obtained was recrystallized from ether to 72 mg. of crystals melting at 208~210°; $[\alpha]_D^{10}$ +120°(c=0.6, $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found : C, 76.71; H, 8.56. U.V. λ_{max}^{MeOH} 241 mμ(ε 18000). I.R. cm^{-1}(in Nujol) : 1730(15-CO), 1701(20-CO), 1661(3-CO), 1613(Δ^4).

(ii) A solution of 81 mg. of (VI) dissolved in 20 cc. of MeOH containing 0.01 g. of KOH was catalytically reduced with 1% Pd-C (0.03 g.). After absorbing about 5.5 cc. of H_2, the hydrogenation was stopped. After removal of the catalyst, the solvent was diluted with water and extracted with $CHCl_3$. After drying over anhyd. Na_2SO_4, $CHCl_3$ was distilled off, the residue so obtained was purified by alumina chromatography, and recrystallized from ether to (IX) as crystals of m.p. 205~208°.

Its infrared spectrum was identical with that of (III) obtained by the method (i). *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found : C, 76.77; H, 8.82.

Summary

The microbiological transformation of progesterone with *Syncephalastrum* sp. was carried out and the dihydroxyprogesterone so obtained was found to have the hydroxyl groups in the 7α- and 15β-positions.

(Received March 7, 1958)

UDC 547.836.7

72. Kyosuke Tsuda und Seitaro Saeki : Die Quaternisierungsreaktion am Ringstickstoff von stereoisomeren Hexahydrojulolidine.

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Die Erfahrungen, die wir bei der Konfigurationsuntersuchung[1] des Allomatrins (I) bzw. Matrins (II) gemacht haben, haben bewiesen, dass das Matrinmolekül, in dem der *trans*-Chinolizidinring (A, B) und der C-Ring auf der 15-, 16- und 17-Stellung miteinander *cis*-verknüpft sind, gegen die Einwirkung von Jodmethyl bzw. Bromcyan intakt bleibt. Es ist also wünschenswert, Verbindungen mit ähnlichem Ringsystem wie Matrin herzustellen und weiter festzustellen, ob durch einen solchen *cis*-verknüpften Ring die Annäherung eines solchen Reagenz an das ungebundene Elektronenpaar des N-4-Ringstickstoffs verhindert wird. In diesem Zusammenhang interessierte uns vor allem das *cis,cis*-Hexahydrojulolidin (IV).

Zur Herstellung des letzteren gingen wir vom Julolidin (VI)[2] aus, welches man nach Protiva-Prelog[3] durch katalytische Hydrierung mittels Platinoxydes oder mittels Raney

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1) K. Tsuda, H. Mishima : Dieses Bulletin, **5**, 285(1957).
2) Org. Syntheses, **26**, 40(1946).
3) M. Protiva, V. Prelog : Helv. Chim. Acta, **32**, 621(1949).