the reaction mixture was refluxed for 1 hr. on a steam bath and allowed to stand at room temperature overnight. The solvents were evaporated and product dissolved in ether and the ether solution washed with water, dried, and concentrated to give the C-20 oxime as a solid, m.p. 216–220°.[18]

Anal. Calcd. for $C_{23}H_{35}O_5N$: C, 68.21; H, 8.69; N, 3.45. Found: C, 68.38; H, 8.91; N, 3.18.

The above oxime (3 g.) was hydrogenated in 30 cc. of acetic acid with 600 mg. of platinum oxide catalyst. The hydrogenation product was filtered, evaporated *in vacuo,* and redissolved in 15 cc. of acetic acid and 45 cc. of water. To the acetic acid solution was slowly added at 0° 5 g. of sodium nitrite dissolved in 5 cc. of water. A gummy oil slowly separated which solidified overnight at room temperature. The solid was extracted with ethyl acetate and the ethyl acetate extract washed free of acid with aqueous potassium bicarbonate solution and dried over magnesium sulfate. The product obtained after evaporation of the solvent was acetylated with pyridine and acetic anhydride and the acetylated product chromatographed on acid-washed alumina. The initial eluate afforded a small amount of the 17a ketone IX. Eluates consisting of 10% to 20% ether in benzene gave an appreciable amount (500 mg.) of a nitrogenous individual (see ref. 9), m.p. 180–183°. Found: C, 65.91, 65.70; H, 8.06 which was not further investigated. The eluates consisting of 5–10% ether in benzene afforded 500–600 mg. of VIII, m.p. 237–240°.

Anal. Calcd. for $C_{23}H_{34}O_4$: C, 73.75; H, 9.15. Found: C, 73.76; H, 9.02.

Treatment of 150 mg. of VIII in 15 cc. of 10% sodium methoxide in methanol and refluxing for 2 hr. afforded, on working up, a crystalline mixture of VII and VIII from ether, m.p. 193–209°. Mixed melting point with material obtained from alkaline decomposition of IIb 190–212°, the infrared spectra of the two samples were identical.

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RAHWAY, N. J.

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(18) Prepared by H. Kuo of these Laboratories.

[MERCK SHARP & DOHME RESEARCH LABORATORIES, DIVISION OF MERCK & CO., INC., RAHWAY, NEW JERSEY]

Microbially Produced 7α- and 7β-Hydroxy-Δ^4-3-keto Steroids

W. J. McALEER, M. A. KOZLOWSKI, T. H. STOUDT, AND JOHN M. CHEMERDA

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Microbial methods for producing 7α- and 7β-hydroxy-Δ^4-3-keto steroids are described. Progesterone and desoxycorticosterone were converted to their 7α-hydroxylated analogs with a *Helminthosporium* culture and to their 7β-hydroxylated analogs with a *Cladiosporium* culture. Characterization of the 7-hydroxy progesterones was effected by conversion to the common intermediate, $\Delta^{4,6}$-pregnadiene-3,20-dione. Assignment of configuration is based on the differential rates of oxidation with chromium trioxide.

In examining a wide variety of microbially produced hydroxyprogesterones we have recently encountered two products which upon treatment with methanolic sodium hydroxide showed the shift in ultraviolet absorption maximum from 240 mμ to 285 mμ characteristic of 7-hydroxy-Δ^4-3-keto steroids.

Characterization of these two isolates as 7-hydroxyprogesterones was accomplished by converting both to Δ^6-progesterone ($\Delta^{4,6}$-pregnadiene-3,20-dione) by dehydrating in methanolic sodium hydroxide. Differentiation was established by the different melting points (7α-, m.p. 227–231°, 7β-, m.p. 188–191°) and the nonidentity of the infrared spectra at the longer wave lengths, particularly bands at 9.78 μ and 11.24 μ present in the 7α-hydroxy spectrum and absent in the 7β-hydroxyprogesterone spectrum.

Evidence for the assignment of configuration for the epimeric 7-hydroxyprogesterone was obtained from a study of their relative rates of oxidation with chromium trioxide. Employing the excellent micromethod recently described by Grimmer[1] we found that the epimer produced by the *Helminthosporium* culture (m.p. 227–231°) oxidized much more rapidly than the epimer produced by the *Cladiosporium* culture (m.p. 188–191°). Grimmer reported that under the conditions used axial hydroxyls react more rapidly than the corresponding equatorial epimers. On this basis we have assigned the 7α-hydroxy configuration to the higher melting epimer and conversely the 7β-hydroxy configuration to the lower melting epimer.

No conclusions as to the configuration of the two epimeric 7-hydroxy progesterones could be derived from their optical rotations since both compounds showed nearly identical rotations.

Careful paper strip chromatography, using the system benzene:cyclohexane/formamide:methanol, was successful in differentiating these two compounds. Two points of interest were noted in this chromatogram: (*a*) The 7β (equatorial) epimer was found to be more mobile (17.0 cm. in 30 hr.) than the 7α (axial) epimer (14.0 cm. in 30 hr.). This is the first example, in the hydroxyprogesterone series, of a violation of Savard's rule[2] which proposes that equatorially hydroxylated steroids are more polar than the axial epimers on paper strip chromatograms with Zaffaroni systems.[3]

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(1) G. Grimmer, *Angew. Chem.*, **69**, 400 (1957).

(2) K. Savard, *J. Biol. Chem.*, **202**, 457 (1953).

(3) A. Zaffaroni, R. B. Burton, and E. H. Keutmann, *Science,* **111**, 6 (1950).

(*b*) The 7β-hydroxy steroid reduced "blue tetrazolium" whereas the 7α-hydroxy epimer did not. It was this differential tetrazolium reduction by the two epimers which first suggested the assignment of configuration which we arrived at finally. From his comprehensive examination of the behavior of various steroids with the tetrazolium reagent Andre Meyer[4] found that 6α-hydroxyprogesterone (equatorial) was able to reduce tetrazolium salts while the 6β-hydroxy epimer was not. This observation suggested that the 7-hydroxy steroid which reduced tetrazolium chloride was the equatorial epimer 7β-hydroxyprogesterone.

We have assigned the 7α-configuration to the 7-hydroxyprogesterone which we obtained from the incubation of progesterone with *Helminthosporium sp.* The similarities of the physical properties of this product and the 7-OH progesterone reported by Fried *el al.*[5] suggests that the same configuration may be assigned to the Fried product. Also, the 7-hydroxy DOC obtained with *Helminthosporium sp.* corresponds in its physical constants to the 7-hydroxy DOC previously described by Meystre *et al.*,[6] to which they had assigned the 7α-configuration.

EXPERIMENTAL

7α-Hydroxylation. A 3.2-l. fermentation with progesterone (0.8 g.) was carried out with a vegative culture of *Helminthosporium sp.* (Merck collection Number I-39) for 48 hr. The culture filtrate was extracted with ethyl acetate and the extract was fractionated on a Super Cel partition column using the system benzene:formamide. The steroidal substrate was recovered in the initial fractions; further development eluted the 7α-hydroxyprogesterone. The development of the column was followed by paper strip chromatography. The column cuts which were shown to contain only the 7-hydroxy steroid were combined and evaporated to dryness. The residue was crystallized twice from benzene (150 mg.), m.p. 227–231°, $[\alpha]_D^{23°}$ +154 (dioxane, C --0.5), λ_{max}^{MeOH} 242, λ_{max}^{Nujol} 2.85 μ (OH), 5.88 μ (20-carbonyl), 5.99 μ (conjugated carbonyl), 6.15 μ (C=C).

Anal. Calcd. for $C_{21}H_{29}O_3$: C, 76.32; H, 9.15. Found: C, 75.83; H, 9.81.

Conversion of 7α-hydroxyprogesterone to Δ^6 progesterone. The isolate was treated with methanolic sodium hydroxide at reflux temperature for 0.5 hr. Recovery of the product was effected by evaporating the methanol and partitioning the residue between water and Skellysolve C. Concentration of the Skellysolve C to a small volume yielded a crystalline solid which was recrystallized from 100% ethanol, m.p. 140–144°, λ_{max}^{MeOH} 285 mμ. Mixed melting point with an authentic sample of $\Delta^{4,6}$-pregnadiene-3,20-dione gave no depression and the infrared spectra of the reaction product and the authentic sample were identical.

The preparation and isolation of Δ^4-pregnene-7α,21-diol-3,20-dione (7α-OH DOC) was exactly analogous to the method described above for 7α-hydroxyprogesterone. The transformation product, upon recovery from the partition column, was crystallized twice from ethyl acetate, m.p. 216–225°, λ_{max}^{MeOH} 240 mμ, $[\alpha]_D^{25}$ +144° ($CHCl_3$, C = 1). From an 800-mg. fermentation 485 mg. of the 7-hydroxy steroid was recovered. Treatment of this isolate with methanolic sodium hydroxide showed the shift in absorption to 285 mμ characteristic of 7-hydroxysteroids.

7β-Hydroxylation. Employing conditions similar to those described in the previous section a 10-l. fermentation of progesterone (2.8 g.) with the culture *Cladiosporium sp.* (Merck collection Number SF-523) yielded 432 mg. of 7β-hydroxyprogesterone. The isolate was recrystallized from ethyl acetate:petroleum ether, m.p. 188–191°, $[\alpha]_D^{25}$ +141° ($CHCl_3$, C = 1) and $[\alpha]_D^{20}$ +158° (dioxane, C = 0.5) λ_{max}^{MeOH} 242 mμ, E% 472, λ_{max}^{Nujol} 2.9 μ (OH), 5.90 μ (20-carbonyl), 6.01 μ (conjugated carbonyl) and 6.16 μ (C = C).

Anal. Calcd. for $C_{21}H_{29}O_3$: C, 76.32; H, 9.15. Found: C, 76.65; H, 9.02.

This isolate too was converted to $\Delta^{4,6}$-pregnadiene-3,20-dione by refluxing with methanolic sodium hydroxide. Identity was established on the basis of m.p. 140–144°, λ_{max}^{MeOH} 285 mμ, nondepression upon mixed melting with an authentic sample and identical infrared spectra.

7β-Hydroxy-DOC (Δ^4-pregnene-7β,21-diol-3,20-dione) (273 mg.) was isolated from 4 l. of broth from a fermentation of desoxycorticosterone (800 mg.) with *Cladiosporium sp.* The compound was recrystallized twice from ethyl acetate, m.p. 178–181.5°, λ_{max}^{MeOH} 240, $[\alpha]_D^{25}$ +151° ($CHCl_3$, C = 1). Treatment of this isolate with refluxing methanolic sodium hydroxide showed the characteristic shift from λ_{max}^{MeOH} 240 mμ to 285 mμ, indicating dehydration to a dienone.

Chromium trioxide oxidation of 7α- and 7β-hydroxyprogesterone. Approximately 1-micromole (0.46 mg.) samples of the two 7-hydroxyprogesterones were dissolved in equal volumes (3 ml.) of glacial acetic acid in Beckman cuvettes. Three micromoles (0.3 mg.) of chromium trioxide was added to each solution and the optical density at 350 mμ measured immediately. At "zero time" both steroid solutions as well as a blank containing only CrO_3 showed optical density reading slightly in excess of 1.00. Readings were taken every 5 min. and immediately a divergence of rate of oxidation of the two epimers could be detected. Finally after 1 hr. the *Helminthosporium* epimer (m.p. 227–231°) had reduced 50% (o.d. 0.5) of the CrO_3 while the *Cladiosporium* epimer (m.p. 188–191°) had reduced only 25% of the CrO_3 (o.d. 0.75). The blank solution still showed an optical density of 1.00.

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