the National Science Foundation, the U. S. Public Health Service, and the Allied Chemical Corp. for providing support for this study.

Experimental

Materials.—The preparation of the six acetoxy ketones has been previously described.[9b] The spectra were run on 20% solutions of the compounds in carbon disulfide containing 1% tetramethylsilane.

N.m.r. Spectra.—The Varian Associates V-4311 high-resolution NMR spectrometer with 12″ electromagnet system, operating at 60 mc., V-K3506 super stabilizer, and V-4365 field homogeneity control was employed. The positions of peaks were calibrated by the audiofrequency side band method[22] using a Hewlett-Packard 200-CD audio oscillator as well as graphical interpolation. The frequencies of the side bands were measured with a Hewlett-Packard 524-B electronic counter. Reported line positions are averages of six to ten spectra; the standard deviation was ±0.3 c.p.s.

(22) J. T. Arnold and M. E. Packard, *J. Chem. Phys.*, **19**, 1608 (1951).

Optical rotatory dispersion (Fig. 2) in methanol: **2α-acetoxycholestane-3-one.** (*c* 0.035): $[\alpha]_{650}$ +97°, $[\alpha]_{589}$ +63°, $[\alpha]_{305}$ +777°, $[\alpha]_{265}$ −628°; **2β-acetoxycholestane-3-one** (*c* 0.103): $[\alpha]_{650}$ +34°, $[\alpha]_{589}$ +43°, $[\alpha]_{290}$ +410°, $[\alpha]_{250}$ +140°; **4α-acetoxycholestane-3-one** (*c* 0.045): $[\alpha]_{650}$ −9°, $[\alpha]_{589}$ +18°, $[\alpha]_{300}$ +417°, $[\alpha]_{260}$ −543°; **4β-acetoxycholestane-3-one** (*c* 0.100): $[\alpha]_{650}$ +18°, $[\alpha]_{589}$ +18°, $[\alpha]_{320}$ + 164°, $[\alpha]_{270}$ − 218°; **3β-acetoxycholestane-2-one** (*c* 0.1775): $[\alpha]_{650}$ +158°, $[\alpha]_{589}$ +214°, $[\alpha]_{305}$ +1520°, $[\alpha]_{286}$ 0°, $[\alpha]_{270}$ −1200°, $[\alpha]_{265}$ −950°; **3α-acetoxycholestane-2-one** (*c* 0.105): $[\alpha]_{650}$ +95°, $[\alpha]_{589}$ +114°, $[\alpha]_{317}$ +647°, $[\alpha]_{296}$ 0°, $[\alpha]_{275}$ −525°, $[\alpha]_{260}$ −220°.

[CONTRIBUTION FROM THE BIOLOGICAL AND CHEMICAL RESEARCH DIVISIONS OF G. D. SEARLE AND CO., CHICAGO, ILL.]

Microbiological Transformations. VI. The Microbiological Aromatization of Steroids

BY R. M. DODSON[1] AND R. D. MUIR

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4-Androstene-3,17-dione (I) was converted to 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) by incubation with species of *Pseudomonas* and *Arthrobacter*. The structure of the 9,10-seco-phenol (III) was established by converting it, through a rational series of reactions, to 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (X), which, in turn, had been prepared *via* the dienone–phenol rearrangement of 1,4-androstadiene-3,17-dione (II). This microbial aromatization of androstenedione (I) resembles, in many respects, the sequences postulated for the conversion of androgenic steroids to estrogens in mammals.

We have recently reported the microbiological conversion of 4-androstene-3,17-dione (I)[2] and of 9α-hydroxy-4-androstene-3,17-dione[2] to 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) by fermentation of these compounds with species of *Pseudomonas*, Searle B20-184, and *Arthrobacter*, Searle B22-9, respectively. More recently, we have discovered that species of *Nocardia* and *Arthrobacter*, as well as other *Pseudomonas sp.* [Searle B40-324 (A.T.C.C. 13261) and Searle B40-327 (A.T.C.C. 13262)] also convert Δ^4-3-keto-steroids to the corresponding 9,10-seco-A-aromatic analogs. Because of the uniqueness of this conversion and the possibility that it closely parallels formation of estrogens from androgenic steroids in mammals,[3] we wish to describe in detail the preparation and proof of structure of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III).[4]

Incubation of 4-androstene-3,17-dione with a species of *Pseudomonas*, B20–184, produced, besides very small quantities of 11α-hydroxy-4-androstene-3,17-dione[5] and 7β-hydroxy-4-androstene-3,17-dione,[6] two different phenolic compounds. The first of these, III, m.p. 123.5–125°, was recognized as a phenol initially by its unique ultraviolet spectrum, $\lambda_{max}^{CH_3OH}$ 280 mμ (ε 2,320). It gave a positive Folin–Denis test,[7] was soluble in dilute alkali, was stable in dilute alkaline solution (under nitrogen) over long periods of time, and could be reprecipitated with carbon dioxide or acetic acid. The infrared spectrum of the compound showed the presence of a hydroxyl group (2.90 μ), a five-membered ring carbonyl group (5.76 μ), a six-membered ring carbonyl group (5.89 μ), and an aromatic ring, probably possessing an isolated hydrogen and two adjacent hydrogen atoms (6.22, 6.63, 11.45 and 12.21 μ).[8] Initial carbon and hydrogen analyses were close to both $C_{18}H_{22}O_3$ and $C_{19}H_{24}O_3$.

All possible structures with an intact steroid nucleus were quickly eliminated by the following reasoning: (1) It was assumed that two of the three oxygen atoms occupied positions corresponding to the positions of the two oxygen atoms in androstenedione. (2) It was assumed that no complex rearrangement had occurred and that the aromatic ring was formed by the breaking of the minimum number (one) of carbon–carbon bonds necessary to such formation. (3) The ultraviolet spectrum of III indicated the absence of a carbonyl group adjacent to the aromatic ring. (4) The ultraviolet spectrum of the dienediol triacetate VI indicated the absence of a carbonyl group beta to

(1) Department of Chemistry, University of Minnesota, Minneapolis 14, Minn.

(2) Preliminary communications of these results appeared in *J. Am. Chem. Soc.*, **80**, 5004, 6148 (1958); previous paper in this series: R. M. Dodson, A. H. Goldkamp and R. D. Muir, *ibid.*, **82**, 4026 (1960).

(3) (a) L. L. Engel, *Cancer*, **10**, 711 (1957); (b) A. S. Meyer, *Biochim. et Biophys. Acta*, **17**, 441 (1955); (c) K. T. Ryan, *J. Biol. Chem.*, **234**, 268 (1959); (d) J. E. Longchampt, C. Gual, M. Ehrenstein and R. I. Dorfman, *Endocrinol.*, **66**, 416 (1960).

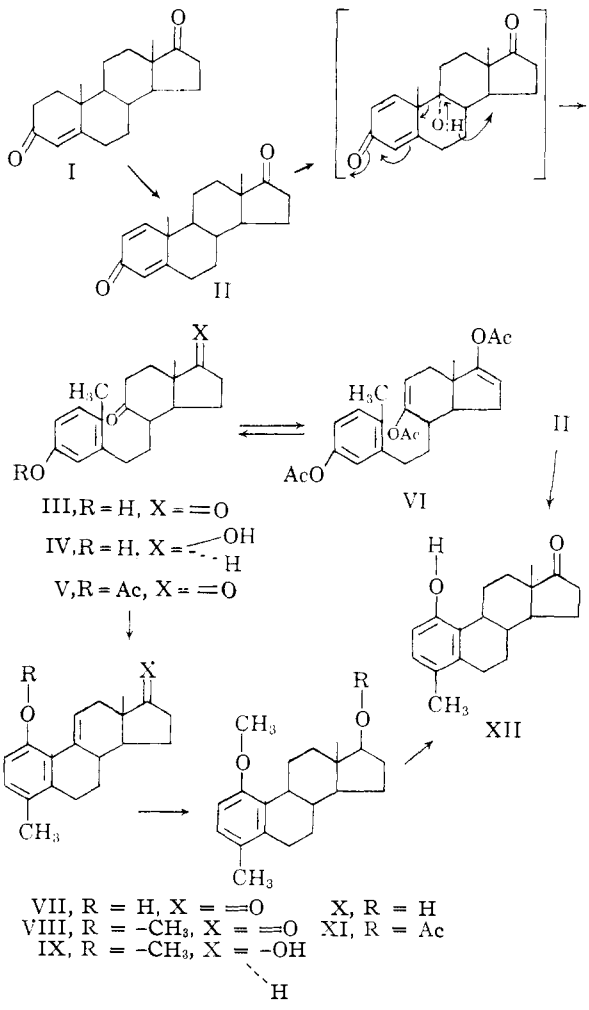
(4) The preparation of the corresponding 9,10-seco-A-aromatic steroids from progesterone, 19-nor-4-androstene-3,17-dione and 3-(3-keto-17β-hydroxy-4-androsten-17α-yl)-propionic acid lactone will be reported later.

(5) S. H. Eppstein, P. D. Meister, H. K. Leigh, D. H. Peterson, H. C. Murray, L. M. Reineke and A. Weintraub, *J. Am. Chem. Soc.*, **76**, 3174 (1954).

(6) R. C. Tweit, A. H. Goldkamp and R. M. Dodson, *J. Org. Chem.*, **26**, 2856 (1961); S. Bernstein, W. S. Allen, H. Heller, R. H. Lenhard, L. I. Feldman and R. H. Blank, *ibid.*, **24**, 286 (1959). This compound was designated 7α-hydroxy-4-androstene-3,17-dione in the latter paper.

(7) O. Folin and W. Denis, *J. Biol. Chem.*, **12**, 239 (1912).

(8) A. S. Dreiding, W. J. Pummer and A. J. Tomasewski, *J. Am. Chem. Soc.*, **75**, 3159 (1953).



the aromatic ring. (5) Stability of III to base indicated the absence of a 12,17-diketone.

The examination of structures in which a bond at C_1–C_{10}, C_9–C_{10}, C_{12}–C_{13} or C_{13}–C_{17} was broken, in conjunction with the statements above and the infrared spectrum, led to the elimination of all reasonable structures except 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) and 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-11,17-dione.[9] Since the 9,10-carbon–carbon bond had been broken in both of these structures, oxidation at C-9 appeared to be more probable than oxidation at C-11. The isolation of 1,4-androstadiene-3,17-dione (II) from a fermentation of androstenedione (I) with *Pseudomonas species*, B40-324,[10] and the paper chromatographic demonstration that androstadienedione (II) was initially formed in this fermentation and then disappeared with the concomitant appearance of the 9,10-seco-phenol (III), led us to believe that the 9,10-seco-phenol (III) was formed *via* the 9α-hydroxylation of the androstadienedione (II), followed by the reverse aldol type reaction depicted above. Consequently, we undertook a definitive proof of structure of the compound believed to be 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III).

3-Acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,-17-dione (V) was simultaneously hydrolyzed and cyclized to 1-hydroxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one (VII) by treatment with methanol–concd. hydrochloric acid (2:1). The position of the double bond in this compound ($\Delta^{9,11}$ rather than $\Delta^{8,9}$) was originally assigned on the basis of the fine structure in its ultraviolet spectrum.[11] This assignment was confirmed by the presence of a band corresponding to an isolated proton on a double bond in the n.m.r. spectrum of the compound (τ = 3.07, 3.20, 3.33, 3.47, two vicinal protons on aromatic ring; τ = 3.75, proton at C-11 on double bond; τ = 4.29, proton on phenolic hydroxyl. The τ-value for the phenolic proton was dependent on concentration).[12] It should be noted that the double bond in VII would be expected to occupy the $\Delta^{9,11}$-position rather than the $\Delta^{8,9}$-position on the basis of the predicted relative stabilities of the two compounds[13] and the direction of dehydration of 9α-hydroxysteroids,[14] irrespective of the stereochemistry of the cyclization reaction.

Compound VII was next converted to 1-methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one (VIII) by means of methyl iodide and potassium carbonate in acetone. Compound VIII, in turn, was reduced to the corresponding 17β-hydroxy-steroid IX using sodium borohydride. The 9,11-double bond in 1-methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17β-ol (IX) was selectively reduced with potassium in liquid ammonia[15] to yield the desired 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (X). This compound X and its acetate XI were identical in all respects (m.p., mixed m.p. and infrared spectra) with a sample of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol, and its acetate, respectively, prepared from 1-hydroxy-4-methyl-1,3,5(10)-estratrien-17-one[16] (XII) *via* methylation and reduction (and acetylation).[17] This sequence of reactions established the structure of the major phenolic product to be 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III).

The second phenolic product, present in only small amount, was very difficult to crystallize, probably because it formed a low melting solvate. It was assigned the structure, 3,17β-dihydroxy-9,10-seco-1,3,5(10)-androstatrien-9-one

(9) Since this work was completed, the preparation of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-11,17-dione, m.p. 208–211°, has been reported; B. J. Magerlein and J. A. Hogg, *Tetrahedron*, **2**, 80 (1958).

(10) *Pseudomonas sp.*, Searle B40-324 (A.T.C.C. 13261), was isolated from Philippine soil and reacted similarly to B20-184 and B40-327.

(11) See, *e.g.*: J. Heer and K. Miescher, *Helv. Chim. Acta*, **31**, 219 (1948).

(12) We are indebted to Dr. N. L. McNevin of the Worcester Foundation for Experimental Biology, Shrewsbury, Mass., for this n.m.r. spectrum.

(13) See, *e. g.*, the arguments advanced by R. B. Turner, W. R. Meador and R. E. Winkler [*J. Am. Chem. Soc.*, **79**, 4122 (1957)] for the stability of double bonds at other positions in steroids.

(14) C. G. Bergstrom and R. M. Dodson, unpublished observations; A. Schubert, D. Onken, R. Siebert and K. Heller, *Chem. Ber.*, **91**, 2549 (1958).

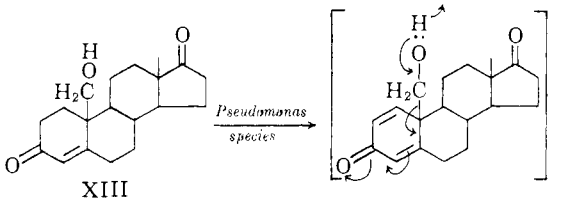
(15) W. S. Johnson, A. D. Kemp, R. Pappo, J. Ackerman and W. F. Johns, *J. Am. Chem. Soc.*, **78**, 6312 (1956).

(16) A. S. Dreiding and A. Voltman, *ibid.*, **76**, 537 (1954).

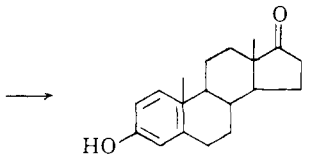
(17) We are indebted to Dr. Willard Hoehn of this Laboratory for the sample of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol acetate, prepared from 1-hydroxy-4-methyl-1,3,5(10)-estratrien-17-one.

(IV), largely on the basis of its analysis, phenolic properties, ultraviolet spectrum and infrared spectrum (2.93, 3.06, 5.87, 6.22, 6.30, 6.675 μ). The difference in molecular rotation between III and IV, ΔM_D (III–IV) = +239°, was in agreement with that expected from the reduction of a 17-carbonyl group to a 17β-ol, ΔM_D(androstenedione[18]-testosterone[18]) = + 228°.

In order to show the correspondence between the aromatization depicted above leading to the 9,10-seco-phenol (III) and an analogous aromatization which may be involved in the conversion of androstenedione (I) to estrone (XIV) in mammals, 19 - hydroxy - 4 - androstene - 3,17 - dione[19] was converted by fermentation with *Pseudomonas sp.*, B40–327, to estrone.[20] It is probable that this con-



XIII



XIV

version also involves the introduction of the Δ^1-double bond followed by a reverse aldol type reaction to give estrone as the final product. Whether or not mammals actually synthesize estrogens from 19-methyl steroids by a process similar to that shown above has not, as yet, been determined.[3] The recent isolation of 19-nor-4-androstene-3,17-dione from the follicular fluid of the mare[21] clearly indicates that alternate pathways are available, but a path through the nor-steroids does not appear to be a probable one.[3c] Although many of the metabolic paths leading to the degradation of steroids by microörganisms have also been demonstrated in mammals, no mammalian degradation *via* 9-hydroxylation and formation of a 9,10-seco-phenol similar to III has, as yet, been uncovered.

Experimental[22]

Fermentation of 4-Androstene-3-17-dione (I). a.—*Pseudomonas sp.* B20–184 was grown as a submerged culture in a stainless steel fermentor in 30 l. of medium containing 200 g. of Difco Nutrient Broth and 2.5 g. of Dow Corning Antifoam AF. The culture was stirred by a paddle type agitator operating at 200 r.p.m. and was aerated with 5 l.p.m. of sterile air through a sparger located below the agitator blades. The temperature was maintained at 25°. Following a growth period of 24 hours, 10 g. of 4-androstene-3,17-dione (I) was added in 250 ml. of acetone and incubation was continued as above for 24 hours. The whole culture was extracted twice, each time with approximately one-half volume of methylene chloride. The extracts were combined and reduced to about 0.5 l.

The methylene chloride solution was warmed on the steam-bath, decolorized with carbon, filtered, then evaporated to dryness. Since attempts at crystallization failed, the 9.63 g. of residue was chromatographed on 1000 g. of silica gel. The column was washed with benzene, 2% ethyl acetate in benzene, 5% ethyl acetate in benzene, and 10% ethyl acetate in benzene. Elution of the column with 15% ethyl acetate in benzene yielded 4.866 g. of material which crystallized only after trituration with ether and melted over a very wide range. Fractional crystallization of one of the fractions from ether–petroleum ether (b.p. 28–38°) yielded III, m.p. 118–122°, and 4-androstene-3,17-dione, m.p. and m.m.p. 171–173°. Since the ultraviolet and infrared spectra of III, as well as the facts that it gave a positive Folin–Denis test[7] and could be dissolved in aqueous sodium hydroxide and reprecipitated with carbon dioxide, indicated that it was phenolic, the remaining fractions eluted with 15% ethyl acetate in benzene were dissolved in ether, and the resulting ether solution extracted with 200 ml. of 5% aqueous sodium hydroxide in four portions. The aqueous solution was filtered, then acidified with concentrated hydrochloric acid. The precipitated product was scratched until crystalline, then separated by filtration to yield 2.77 g. of III, m.p. 119–122°. Crystallization of a portion of this material from ether plus a little acetone, then from ether yielded pure 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III), m.p. 123.5–125°, $[\alpha]_D$ + 100.5°, λ_{max} 280 mμ (ϵ 2,320); λ_{max}^{KBr} 2.90, 5.76, 5.89, 6.22, 6.63, 8.18, 8.77, 11.425–11.475 and 12.21 μ.

Anal. Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 76.14; H, 8.19.

Evaporation of the above ether extract and crystallization of the residue from ether yielded 0.78 g. of 4-androstene-3,17-dione, m.p. and m.m.p. 172–173.5°. No 1,4-androstadiene-3,17-dione was isolated from this initial fermentation, probably because of the isolation procedure used.

The column was next washed with 20% ethyl acetate in benzene and with 25% ethyl acetate in benzene. Elution with 30% ethyl acetate in benzene yielded 321 mg. of material, which was further purified by extraction from ether solution with 5% aqueous potassium hydroxide, liberated from the basic solution by acidification with hydrochloric acid and finally extracted with ether. Attempts at crystallization of the 0.22 g. of product so obtained were unsuccessful. After an unsuccessful attempt to crystallize the acetylated material, it was found that the material, liberated from the diacetate by saponification, would crystallize on long standing with intermittent scratching. Two crystallizations of the product so obtained from ether yielded 86 mg. of 3,17β-dihydroxy-9,10-seco-1,3,5(10)-androstatrien-9-one (IV), m.p. 68–72°, with bubbling. The resolidified melting point sample and the analytical sample, after being dried under high vacuum, melted at 133–134°, $[\alpha]_D$ 15.9°, λ_{max} 279 mμ (ϵ 2,600); λ_{max}^{KBr} 2.93, 3.06, 5.87, 6.22, 6.30 and 6.675 μ.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.71; 75.42; H, 8.80, 8.55.

The column was washed further with 50% ethyl acetate in benzene. Elution with 60% ethyl acetate in benzene yielded 91 mg. of crystalline material. This material was crystallized twice from ether to yield 13.9 mg. of crude 11α-hydroxy-4-androstene-3,17-dione, m.p. 212–216°. This material was identified by comparison of its infrared spectrum with that of an authentic sample of 11 α-hydroxy-androstenedione.[5]

Further elution of the column with 70% ethyl acetate in benzene gave 91 mg. of crystalline material, which after crystallization from acetone–cyclohexane, then aqueous methanol, left 8.9 mg. of 7β-hydroxy-4-androstene-3,17-

(18) Rotations from J. P. Mathieu and A. Petit, "Tables de Constantes et Donees Numeriques 6. Constantes Selectionees Pouvir Rotatoire Naturel, I. Steroids," Masson et Cie., Editeurs, Paris, 1956.

(19) A. S. Meyer, *Experientia*, **11**, 99 (1955).

(20) It should be noted that the sequence of reactions (hydroxylation and dehydrogenation) differs in the aromatization of 19-hydroxy-4-androstenedione (to estrone) and of 4-androstenedione (to the 9,10-seco-phenol, III) by *Pseudomonas sp.* Aromatizations (to the 9,10-seco-phenol, III) involving initial hydroxylation followed by dehydrogenation have been demonstrated with *Nocardia sp.* (ref. 2).

(21) R. V. Short, *Nature*, **188**, 232 (1960).

(22) All melting points were taken on a Fisher–Johns melting point apparatus. The rotations were taken in chloroform at 24 ± 2°, and the ultraviolet spectra in methanol. We are indebted to Drs. R. T. Dillon and H. W. Sause of the Analytical Division of G. D. Searle & Co. for the analytical and optical data reported and to Dr. E. G. Daskalakis for paper chromatographic analyses.

dione, m.p. 221–225°. This material was also identified by comparison of its infrared spectrum with that of an authentic sample.[6]

Further elution of the column yielded no other products.

b. *Pseudomonas sp.* **B40-324 (A.T.C.C. 13261).**—Incomplete fermentation of 20 g. of 4-androstene-3,17-dione (I) with *Pseudomonas sp.* B40-324 yielded 2.35 g. of the 9,10-seco-phenol, III (isolated by alkaline extraction, but purified by crystallization of its acetate). The residue from the ether solution, left after alkaline extraction, was chromatographed on silica gel. From the initial crystalline fractions (4.68 g.) eluted with 10% ethyl acetate in benzene, 3.28 g. of pure 4-androstene-3,17-dione, m.p. and m.m.p. 173–174.5°, was obtained by crystallization from a mixture of ether and acetone. From the crystalline fractions (1.44 g.) eluted with 10% ethyl acetate, after elution of 4-androstene-3,17-dione, 0.86 g. of pure 1,4-androstadiene-3,17-dione (II), m.p. 142–143.5°, was obtained by crystallization from ether containing a little acetone. The androstadienedione was identified by comparison of its infrared spectrum with that of an authentic sample and by the lack of melting-point depression of a mixture of this material and an authentic sample.

c. *Arthrobacter* **sp. B22-8 (A.T.C.C. 13260)** was cultivated in submerged culture as described above for *Pseudomonas sp.* B20-184 for 17 hours. 4-Androstene-3,17-dione (I) (10.0 g.) was added in 350 ml. of acetone and incubation was continued for 7 hours. The whole culture was extracted with methylene chloride.

The methylene chloride solution was evaporated to dryness and the 9,10-seco-phenol, III, separated by alkaline extraction from ether. Purification by crystallization of its acetate from methanol yielded 1.10 g. of 3-acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (V), m.p. 144–145.5°, identical (m.m.p., infrared spectrum) with the sample described below. Chromatography of the residue from the ether extract yielded 4-androstene-3,17-dione, m.p. and m.m.p. 172–173°, and 0.75 g. of pure 1,4-androstadiene-3,17-dione, m.p. 141–143° (m.m.p. and infrared spectrum).

3-Hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III).—A sample of crude 9,10-seco-phenol, III, isolated by alkaline extraction, was carefully chromatographed on silica gel. Crystallization of the bulk of the material, eluted with 10% ethyl acetate in benzene, from acetone–cyclohexane yielded 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III), m.p. 133.5–134.5° $[\alpha]_D$ + 97.3°. The infrared spectrum of this material in KBr differed from that described above, but their infrared spectra in chloroform solution were identical. Acetates prepared from the two samples were also identical. From this we concluded that the 9,10-seco-phenol, III, exists in two polymorphic forms.

Anal. Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 76.08; H, 8.30.

3-Acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (V).—A solution of 0.50 g. of the 9,10-seco-phenol. III, in 2.0 ml. of pyridine and 2.0 ml. of acetic anhydride was allowed to stand overnight at room temperature. The resulting solution was filtered to free it from a small quantity of insoluble material, and the residue was washed with 2.0 ml. of pyridine. The filtrate was stirred with ice and water until crystals formed, then the product was separated by filtration; m.p. 110–111.5°. Crystallization of this material from dilute acetone gave 0.52 g. of 3-acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione, m.p. 145.5–147.5°. Two crystallizations from methanol raised the m.p. of this product to 147–147.5°, $[\alpha]_D$ + 82.5°; λ_{max} 266 mμ (ε 650), 273 mμ (ε 650); λ230 (ε 2,530); λ_{max}^{KBr} 5.70, 8.225 μ (acetate), 5.77 μ (carbonyl on 5-membered ring), 5.90 μ (carbonyl on 6-membered ring); 6.225, 6.325, 6.71, 11.10, 11.97 μ (aromatic ring and attached hydrogens).

Anal. Calcd. for $C_{21}H_{26}O_4$: C, 73.67; H, 7.66. Found: C, 73.34; H, 7.61.

3,9,17-Triacetoxy-9,10-seco-1,3,5(10),9(11),16-androstapentaene (VI).—A solution of 0.50 g. of the 9,10-seco-phenol acetate, V, and 0.50 g. of *p*-toluenesulfonic acid in 20.0 ml. of isopropenyl acetate was heated under reflux for 5 hours using an air condenser and holding the condensation ring 2 inches from the top of the condenser. The volume of the reaction mixture was then decreased by distillation and 0.50 g. of anhydrous sodium acetate was added. The resulting suspension was diluted with water and extracted with ether. The solution was washed with dilute aqueous sodium bicarbonate, then water; it was then evaporated to dryness. The product was dissolved in cyclohexane and filtered through 5 g. of Florisil in a sintered glass funnel. The Florisil was washed with two portions of cyclohexane, then three portions of benzene. Very little product was present in the last benzene wash. The fractions were then combined and evaporated to dryness. The 0.47 g. of material so obtained failed to crystallize. A 0.127-g. sample was dried at 111° under high vacuum, then submitted for analysis; $\epsilon_{273m\mu}$ 1,170, $\epsilon_{266m\mu}$ 1,250, $\epsilon_{230m\mu}$ 4,740; $\lambda_{max}^{CHCl_3}$ 5.72, 6.70, 7.32, 8.09 and 8.40 μ.

Anal. Calcd. for $C_{25}H_{30}O_6$: C, 70,40; H, 7.09. Found: C, 70.42; H, 7.44.

In order to eliminate the possibility that cyclization or some unexpected rearrangement had occurred during the enol acetylation, a small portion of the above dienediol triacetate, VI, was hydrolyzed, using methanolic potassium hydroxide at room temperature, back to the starting 9,10-seco-phenol, III (identity established by m.m.p. and comparison of infrared spectra).

1-Hydroxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one (VII).—A solution of 0.88 g. of the 9,10-seco-phenol acetate, V, m.p. 145–146.5°, in 20 ml. of methanol was treated with 10 ml. of concd. hydrochloric acid. The air was displaced from the flask with nitrogen; the flask was stoppered and was allowed to stand at room temperature for 2.5 days. The beautiful precipitate which formed was separated by filtration, washed on the filter with a little ether, then with water, and finally dried at room temperature to yield 0.65 g. (90%) of 1-hydroxy-4-methyl-1,3,5-(10),9(11)-estratetraen-17-one (VII), m.p. 194–197°. Crystallization of a small portion of this material from acetone plus cyclohexane failed to raise the melting point, 193.5–197°, $[\alpha]_D$ +264°; λ_{max} 255.5 mμ (ε 13,200), 300 mμ (ε 3,830); $\lambda_{shoulders}$ 310 mμ, 265 mμ; λ_{max}^{KBr} 3.07, 5.79, 6.18, 6.29, 6.84 μ.

Anal. Calcd. for $C_{19}H_{22}O_2$: C, 80.81; H, 7.86. Found: C, 80.55; H, 7.73.

1-Methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one (VIII).—A solution of 0.58 g. of 1-hydroxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one in 25 ml. of acetone was treated with 5.0 ml. of methyl iodide and 5.52 g. of anhydrous potassium carbonate. The resulting suspension was heated under reflux with stirring for approximately 18 hours. The reaction mixture was then evaporated to dryness under a jet of nitrogen; water was added to the residue; the resulting suspension was stirred thoroughly; then the product was separated by filtration and washed thoroughly with water. Crystallization of this material from dilute methanol yielded 0.44 g. of 1-methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17-one (VIII), m.p. 123–125°. Recrystallization from petroleum ether, b.p. 60–70°, gave rose-colored crystals of the same melting point; $[\alpha]_D$ + 263.5°; λ_{max} 218 mμ (ε 28,900), 257 mμ (ε 13,000), 297 mμ (ε 3,150), shoulders 267 and 306 mμ.

Anal. Calcd. for $C_{20}H_{24}O_2$: C, 81.04; H, 8.16. Found: C, 80.91; H, 8.22.

1-Methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17β-ol (IX).—A solution of 0.37 g. of 1-methoxy-4-methyl-1,3,5-(10),9(11)-estratetraen-17-one in 10 ml. of methanol was treated with 0.37 g. of sodium borohydride in 2 ml. of water and 8 ml. of methanol. After the resulting solution had stood at room temperature for 15 minutes, the excess sodium borohydride was decomposed by the cautious addition of dilute acetic acid. By heating this solution on the steam-bath and diluting it with water to the point of incipient crystallization, we obtained directly 0.37 g. of pure 1-methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17β-ol (IX), m.p. 144–145.5° (unchanged by recrystallization from petroleum ether, b.p. 60–70°), $[\alpha]_D$ + 161.2°; λ_{max} *ca.* 220 mμ (ε 26,350), 258 mμ (ε 13,230), 297 mμ (ε 3,110), shoulders 267 and 306 mμ.

Anal. Calcd. for $C_{20}H_{26}O_2$: C, 80.49; H, 8.78. Found: C, 80.38; H, 8.95.

1-Methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (X).—A solution of 0.30 g. of 1-methoxy-4-methyl-1,3,5(10),9(11)-estratetraen-17β-ol (IX) in 5.0 ml. of anhydrous ether, and 30.0 ml. of liquid ammonia was treated with 0.35 g. of potassium metal and stirred for 20 minutes. Then a mixture of 2.0 ml. of absolute ethanol and 2.0 ml. of anhydrous

ether was added dropwise over a period of 5 minutes. The ammonia was allowed to evaporate and the other solvents were removed under a jet of nitrogen. The residue was dissolved in ether; the resulting solution was washed with water, decolorized with carbon, dried (sodium sulfate), filtered, and the solvent evaporated. Crystallization of the residue from dilute methanol, then twice from acetone–petroleum ether, b.p. 60–70°, yielded 0.12 g. of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (X), m.p. 115–116.5°. Further crystallization from petroleum ether, b.p. 60–70°, raised the m.p. to 116.5–117.5°, $[\alpha]_D$ + 185.3°; λ_{max} 278 mμ (ε 1,740), 285 mμ (ε 1,760). This material proved to be identical (m.m.p. and infrared spectra) to a sample of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol,[17] m.p. 118–118.5°, prepared from 1-hydroxy-4-methyl-1,3,5(10)-estratrien-17-one[16] (which in turn was prepared from 1,4-androstadiene-3,17-dione (II) *via* the dienonephenol rearrangement).

Anal. Calcd. for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 80.14; H, 9.67.

Acetylation of 50.2 mg. of the above product with acetic anhydride and pyridine, followed by crystallization of the acetate from methanol, yielded 33.5 mg. of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol acetate (XI), m.p. 148.5–150°. This material was identical (m.m.p. and infrared spectra) with an authentic sample[17] prepared *via* the dienol–phenol rearrangement of 1,4-androstadiene-3,17-dione (II).

Estrone from 19-Hydroxy-4-androstene-3,17-dione.—*Pseudomonas sp.* B40-327 (A.T.C.C. 13262) was grown as a submerged culture for 24 hours in 100 ml. of Difco Nutrient Broth in a 500-ml. erlenmeyer flask. Incubation was at 25° on a rotary shaker operating at 200 r.p.m. through a 2-inch diameter revolution. 19-Hydroxy-4-androstene-3,17-dione (25 mg.) was added to the culture in 1 ml. of acetone and incubation was continued for an additional 24 hours. The culture was extracted twice with methylene chloride and the rich solvent extracts were pooled and reduced to dryness. Paper chromatographic analysis of this residue indicated a 70–80% yield (estimated visually) of material behaving like estrone.

The residue from the fermentation was dissolved in ethyl acetate. The solution was filtered from a small quantity of insoluble material, then evaporated to dryness. Crystallization of the residue from acetone–petroleum ether, b.p. 60–70°, then from dilute acetone yielded 1.4 mg. of estrone (XIV), m.p. 259–262°. Comparison of the infrared spectrum of this material with that of an authentic sample of estrone confirmed identity.

Since the yield of estrone obtained by direct crystallization was so small, all of the mother liquors from the above crystallization were combined, then evaporated to dryness. The residue was dissolved in a mixture of ether and benzene and the estrone isolated by alkaline extraction. In this way an additional 4.6 mg. of estrone, m.p. 259–262°, was obtained. In order to purify it, this estrone was sublimed at 200° and 0.2 mm. pressure. After sublimation this material showed no depression in melting point when mixed with an authentic sample, m.p. and m.m.p. 260–262°

[CONTRIBUTION FROM THE BIOLOGICAL AND CHEMICAL RESEARCH DIVISIONS OF G. D. SEARLE AND CO., CHICAGO 80, ILL.]

Microbiological Transformations. VII. The Hydroxylation of Steroids at C-9

BY R. M. DODSON[1] AND R. D. MUIR

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Incubation of 4-androstene-3,17-dione (I) with *Nocardia sp.* produced 9α-hydroxy-4-androstene-3,17-dione (II) and 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III). The structure of the 9α-hydroxyandrostenedione (II) was established by converting it by fermentation with a species of *Arthrobacter* to the known 9,10-seco-phenol (III). Incubation of progesterone (IV) with *Nocardia sp.* produced 9α-hydroxyprogesterone (V) and 9α-hydroxytestosterone (VI). The structure of the 9α-hydroxyprogesterone (V) was established by its conversion by fermentation with a species of *Arthrobacter* to 9α-hydroxyandrostenedione (II). 9α-Hydroxytestosterone was synthesized by the sodium borohydride reduction of 9α-hydroxyandrostenedione (II). The configuration of the 9α-hydroxyl group in these steroids was determined by the conversion of 9α-hydroxyprogesterone (V) to 3β-hydroxy-3α,9α-epoxy-5β-pregnan-20-one (XIII) on reduction.

We have recently reported the preparation of 3-hydroxy - 9,10 - seco - 1,3,5(10) - androstatriene-9,17-dione (III)[2] by the incubation of 4-androstene-3,17-dione (I) with a species of *Pseudomonas*. Knowledge of the structure of this compound, III, enabled us to establish definitively the structure of 9α-hydroxy-4-androstene-3,17-dione (II), 9α-hydroxytestosterone (VI) and 9α-hydroxyprogesterone (V) obtained from fermentations using species of *Nocardia*.[3]

Fermentation of 4-androstene-3,17-dione (I), by the methods previously described,[4] with a species of *Nocardia*, A.T.C.C. 13259, isolated from soil, produced 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) and a monohydroxy-4-androstene-3,17-dione (II), m.p. 222–223.5°. The ultraviolet and infrared spectra of compound II confirmed the presence of the new hydroxyl group, of the 3-keto group conjugated with the 4,5-double bond and of the 17-keto group. The failure of compound II to acetylate, when treated with pyridine and acetic anhydride, indicated that the hydroxyl group occupied either a tertiary position (C-8,9 or 14) or the 11β-position. This information, in conjunction with the isolation of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) from the fermentation, made the 9-position the most probable site for the new hydroxyl group.

Fermentation of compound II with a species of *Arthrobacter* (Searle B22-9), known to convert 4-androstene-3,17-dione to 1,4-androstadiene-3,17-dione in excellent yield, gave 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III). The latter compound was purified as its acetate, which proved to be identical in all respects with the 3-acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione reported previously.[2] Thus, compound II was proved to be 9-hydroxy-4-androstene-3,17-dione.[5]

(1) Department of Chemistry, University of Minnesota, Minneapolis 14, Minn.

(2) R. M. Dodson and R. D. Muir, *J. Am. Chem. Soc.*, **83**, 4627 (1961); R. M. Dodson and R. D. Muir, *ibid.*, **80**, 5004 (1958).

(3) A preliminary communication on the 9α-hydroxylation of androstenedione by *Nocardia sp.*, A.T.C.C. 13259 (G. D. Searle A20-10) appeared in the *J. Am. Chem. Soc.*, **80**, 6148 (1958). Since the completion of the work described in this paper, the conversion of progesterone to 9α-hydroxyprogesterone using a species of *Nocardia* has been reported by C. J. Sih and F. L. Weisenborn, *ibid.*, **82**, 2653 (1960).

(4) D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister and H. M. Leigh, *ibid.*, **74**, 5933 (1952).

(5) The specific rotation of the previously described 8β(or 9α)-hydroxy-4-androstene-3,17-dione, m.p. 214–217°, $[\alpha]^{23}_D$ 165° ($CHCl_3$),