condensation and its pentacetylated malononitrile derivative is given as compound IV. These two compounds serve as confirmatory proof of the course of the reaction.

Methoxymethylation of pyrones by chloromethyl ether in the presence of trifluoroacetic acid is described and the compounds produced are given in chart III as members of the IIIa–d series.

The results given show that mono- or di-methoxymethylation may take place depending upon the mole ratios of the reactants. The probable structures of the compounds produced are reflected in the names assigned to the members of the series

EXPERIMENTAL[6]

Preparation of compounds of Ia–f *series.* A mixture consisting of 0.1 mole of the pyrone, 0.1 mole of the acid, and 20 ml. of trifluoroacetic acid was refluxed for 15 hours. The reaction mixture was then diluted with 60–80 ml. of water and chilled in the freezing compartment of the refrigerator. The precipitate was filtered off and dried in air to give the crude yields listed in Table I. Compound If required 0.2 mole of the organic acid to 0.1 mole of the pyrone and 25 ml. of trifluoroacetic acid was used.

The analytical samples were obtained in the following manner: Ia and Ib, recrystallized twice from ethanol; Ic, recrystallized three times from ethanol; Id and Ie, recrystallized twice from heptane; If recrystallized once from ethanol and once from heptane.

Preparation of compounds of IIa–d *series.* One tenth mole of the pyrone was mixed with 0.1 mole of the ester and 20 ml. of trifluoroacetic acid and the mixture refluxed for 15 hours. At the termination of the reaction period 40 ml. of absolute ethanol was added and the mixture was allowed to stand in the freezer for at least 12 hours. The precipitates were dried in air.

The analytical samples of the several compounds were obtained in the following manner: IIa, recrystallized twice from ethanol then twice from heptane; IIb, extracted with heptane then recrystallized from ethyl acetate; IIc, recrystallized 3 times from ethanol; IId, recrystallized once from ethanol.

Preparation of compounds of the IIIa–d *series.* Fifteen milliliters of chilled trifluoroacetic acid was mixed with 0.1 mole of the pyrone and 0.1 mole of cold chloromethyl ether. The mixture was gently heated, in an efficient all glass reflux assembly in a glass heating mantle. The heating was continued until hydrogen chloride vapors were no longer evolved (usually complete in about an hour). The reaction mixture was diluted, in each case, with 40 ml. of absolute ethanol and chilled. The precipitated compound was filtered off and dried in air.

Analytical samples were obtained by recrystallizing the compounds twice from ethanol.

The synthesis of compound IV. A 3-g. sample of IIE was refluxed for 90 minutes with 25 ml. of acetic anhydride and 4 g. of malononitrile. The mixture was then diluted with water, chilled, and filtered. The compound was dried in air to give a brown material of the pentaacetylmalononitrile derivative, yield 60%. The compound was recrystallized once from heptane, m.p. dec. above 195°.

Anal. Calcd. for $C_{26}H_{19}BrN_2O_{11}$: C, 50.74; H 3.11; N, 4.55. Found: C, 50.52; H, 3.02; N, 4.38.

(6) All analyses were performed by Dr. Carl Tiedcke, Teaneck, N. J. and all melting points were taken on a Fisher-Johns melting point assembly.

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Microbiological Transformations. VIII. The Oxidation of Androstenedione at C-16

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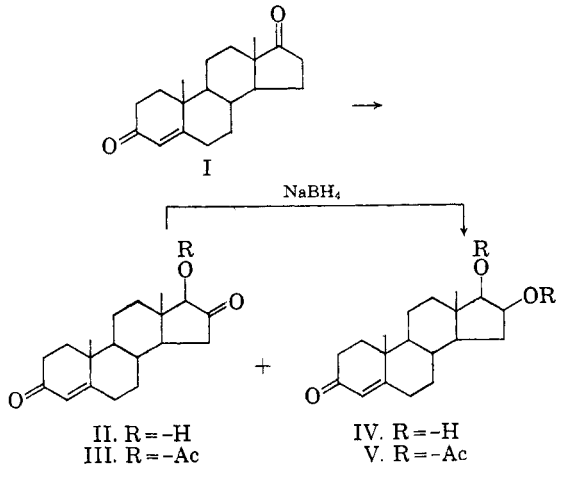
The recently reported microbiological hydroxylation of 17α,21-dihydroxy-4-pregnene-3,20-dione (Reichstein's compound S) at C-19 using *Corticium sasakii*[2] prompted us to investigate the oxidation of 4-androstene-3,17-dione (I) with this same genus. A simple preparation of 19-hydroxyandrostenedione would be most useful to the commercial production of the biologically active norsteroids,[3] 17α-ethyl-19-nortestosterone and 17α-ethynyl-17β-hydroxy-19-nor-5(10)-androsten-3-one.



Incubation of 4-androstene-3,17-dione (I) with *Corticium centrifugum*, A.T.C.C. 11908, produced 16-ketotestosterone (II) and 16β-hydroxytestosterone (IV). The position of oxidation in II was readily determined by the fact that the compound gave a positive blue-tetrazolium test,[4] but showed no appreciable change in its ultraviolet spectrum over a 24-hour period when allowed to stand in 0.1*N* methanolic potassium hydroxide.[5] These

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tests clearly indicated hydroxylation adjacent to a carbonyl group but no hydroxylation in rings A or B of the androstenedione (I). The 16-ketotestosterone (II) was positively identified by the excellent correspondence of its physical properties and those of its 17-acetate (III) with the physical properties of the same compounds previously described by Adams *et al.*[6] The isomeric 16α- and 16β - hydroxy - 4 - androstene - 3,17 - diones were eliminated as possible structures of II by an analysis of the expected molecular rotations of the compounds (see ref. 6c) and by the recovery of II unchanged from alkaline solution.[7]

The structure of 16β-hydroxytestosterone (IV) was initially assigned on the basis of the following facts: (1) The Δ^4-3-keto group was still intact (ultraviolet and infrared spectra) but the 17-carbonyl group was no longer present. (2) The compound gave a positive test with lead tetraacetate in benzene. (3) The physical properties of IV and its diacetate (V) differed from those reported by Adams *et al.*[8a] for 16α-hydroxytestosterone and its diacetate, but the melting point of V agreed well with that previously reported by Butenandt *et al.*[8] for 16β-hydroxytestosterone diacetate. The structure of IV was established by its synthesis from 16-ketotestosterone by the selective reduction of the 16-carbonyl group with sodium borohydride.[9] The configuration of the 16β-hydroxyl group in IV was confirmed by the ready conversion of IV to the corresponding acetonide.[6c,8]

The isolation of 16β-hydroxytestosterone (IV) from the incubation of androstenedione (I) with *C. centrifugum* does not necessarily indicate that this organism directly hydroxylates any substrate at the 16β- position. 16α-Hydroxylation of androstenedione (I), followed by isomerization of the 16,17-ketol (to II)[7] and reduction of the resulting 16-keto group (to give IV) provides as adequate an explanation of the compounds obtained as a sequence involving 16β-hydroxylation of androstenedione and/or testosterone. At present, we do not know the detailed course of the reaction.

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EXPERIMENTAL[10]

Fermentation of 4-androstene-3,17-dione (I) with Corticium centrifugum, A.T.C.C. 11908. *Corticium centrifugum*, A.T.C.C. 11908, was grown for 90 hr. in a stainless steel fermentor containing the following medium: 1000 g. of commercial dextrose, 150 g. of cotton seed flour, 90 ml. of corn steep liquor, 20 g. of yeast extract, 5 g. of silicone antifoam, and water to a total volume of 35 l. after sterilization with direct steam. The culture was agitated by a paddle type stirrer operating at 200 r.p.m. and was aerated with 10 l.p.m. of sterile air which entered through a sparger located below the agitator. The temperature was maintained at 25°. Ten grams of 4-androstene-3,17-dione dissolved in 250 ml. of acetone was added, and incubation was continued for 7 hr. The culture was extracted twice with 15 l. of methylene chloride, and the rich solvent fractions were pooled.

The methylene chloride solution was evaporated to dryness, and the residue chromatographed on 1.5 kg. of silica gel. The column was first washed with 10% ethyl acetate in benzene. Elution of the column with 15% ethyl acetate in benzene produced 2.686 g. of 4-androstene-3,17-dione, m.p. and m.m.p. 172–174°. The column was washed further with 20% ethyl acetate in benzene and 30% ethyl acetate in benzene. Elution with 40% ethyl acetate in benzene yielded 1.734 g. of 16-ketotestosterone. Crystallization of this material from acetone–cyclohexane, aqueous acetone, then from acetone–petroleum ether (b.p. 60–70°) gave 604 mg. of 16-ketotestosterone (II), m.p. 161–162.5°; $[\alpha]_D$ −66°; λ_{max} 240 mμ (ϵ 16,350); λ_{max}^{KBr} 2.90, 5.70, 5.98, 6.18 μ; (reported[6a] m.p. 160°, $[\alpha]_D$ −65°). An additional 0.48 g. (m.p. 157–159°) and 0.14 g. (m.p. 153–157°) of 16-ketotestosterone were obtained from the mother liquors. The compound gave an intense purple color on standing with conc. sulfuric acid.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.62; H, 8.68.

Further elution of the column with 50% ethyl acetate in benzene yielded 715 mg. of crude 16β-hydroxytestosterone, which, since it proved difficult to purify, was crystallized in two portions. Crystallization of the first three fractions (369 mg.) from acetone–cyclohexane, aqueous methanol, acetone–petroleum ether (b.p. 60–70°), aqueous methanol, then finally aqueous acetone yielded 59 mg. of 16β-hydroxytestosterone (IV), m.p. 183.5–185.5°; $[\alpha]_D$ +103°; λ_{max} 241 mμ (ϵ 16,800); $\lambda_{max}^{CHCl_3}$ 2.73, 2.81, 5.99, 6.19, 9.37, and 9.65 μ (reported[6c] m.p. 179–182°, $[\alpha]_D$ +101°). Crystallization of the final four fractions (346 mg.) yielded 89.5 mg. of product, m.p. 178–183°. An additional 129 mg. of product, m.p. 168–178°, was obtained from the mother liquors.

Anal. Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.73; H, 9.17.

Further elution of the column with 60% and 70% ethyl acetate in benzene, then finally with ethyl acetate, yielded no further crystalline material.

16-Ketotestosterone acetate (III) was obtained by acetylation of 16-ketotestosterone with acetic anhydride in pyridine for 2 hr. at room temperature. After crystallization from dilute acetone the material melted at 200–201.5°; $[\alpha]_D$ −48°; λ_{max} 240 mμ (ϵ 17,000); (reported[8a] m.p. 196–198°, $[\alpha]_D$ − 48°).

Anal. Calcd. for $C_{21}H_{28}O_4$: C, 73.23; H, 8.19. Found: C, 73.39; H, 8.23.

16β-Hydroxytestosterone diacetate (V) was obtained by acetylation of 16β-hydroxytestosterone (IV) with acetic anhydride in pyridine for 18 hr. at room temperature.

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After crystallization from acetone–petroleum ether (b.p. 60–70°) the material melted at 204–205.5°; $[\alpha]_D$ +90°; λ_{max} 240 mμ (ε 16,900); (reported[6e] m.p. 201–202.5°, $[\alpha]_D$ +88°).

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.16; H, 8.05.

16β-Hydroxytestosterone (IV) *from 16-ketotestosterone* (II). To a solution of 0.50 g. (1.65 mmole) of 16-ketotestosterone in 100 ml. of ethanol, cooled to 4° in an ice bath, was added a solution of 0.10 g. (2.64 mmole) of sodium borohydride in 5 ml. of water. The resulting solution was allowed to stand at 0–5° for 1 hr. Then 1.00 ml. of glacial acetic acid was added to decompose the excess sodium borohydride, and the resulting solution was evaporated to dryness. Attempts at purification of the product by direct crystallization from dilute acetone, acetone–petroleum ether (b.p. 60–70°) and dilute acetone, gave material melting at 173.5–175°. Acetylation of this material plus material recovered from the mother liquors from the crystallizations (0.382 g. total) with acetic anhydride (5 ml.) in pyridine (5 ml.) yielded, after crystallization from dilute acetone, then acetone–petroleum ether (b.p. 60–70°), 204 mg. of 16β-hydroxytestosterone diacetate (V), m.p. 198–203°. A final crystallization from acetone–petroleum ether (b.p. 60–70°) gave material, m.p. 203–204.5°, $[\alpha]_D$ + 89°, that was identical in all respects (m.m.p. and infrared spectra) with that prepared above.

Hydrolysis of 153 mg. of 16β-hydroxytestosterone diacetate (V) with 0.20 g. of sodium hydroxide in 1 ml. of water and 7 ml. of methyl alcohol under nitrogen (room temperature, 18 hr.) gave, after crystallization from dilute methanol, 96.5 mg. (81%) of 16β-hydroxytestosterone, m.p. 180–182.5°. After crystallization from dilute acetone, this material, m.p. 183–184.5°, proved to be identical in all respects (m.m.p. and infrared spectra) with that obtained directly from the fermentation.

16β-Hydroxytestosterone acetonide was prepared by treating a solution of 16β-hydroxytestosterone (46 mg.) in 5 ml. of acetone with *p*-toluenesulfonic acid (0.10 g.) for 18 hr. The acid was neutralized with solid potassium carbonate. The product was precipitated with water and crystallized from dilute acetone, m.p. 183.5–187° (cloudy melt); $\lambda_{max}^{CHCl_3}$ 5.98, 6.18, 7.22, 7.26, 9.42, and 11.53 μ.[11]

Anal. Calcd. for $C_{22}H_{32}O_3$: C, 76.70; H, 9.36. Found: C, 76.98; H, 9.25.

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Communications to the Editor

High Pressure Gas Chromatography above Critical Temperatures[1]

Sir:

In studies on the separation of porphyrin mixtures by conventional gas chromatography, sufficiently high vapor pressures were reached only at temperatures where decomposition occurred. Higher vapor concentrations at lower temperatures are necessary for separations. For thermodynamic reasons liquids show higher vapor tension under pressure from insoluble gases.[2-4] This increase is relatively small. Inorganic and organic solids,[3-9] including a derivative of chlorophyll,[5] are soluble in carbon dioxide, ammonia, sulfur dioxide, ethanol, and ether far in excess of the thermodynamic increase in vapor tension. Solubility increases with pressure and van der Waals' interaction in the gas. Later the dissolution of polymers was found to be common.[10]

In our apparatus (see Fig. 1) the gas is contained in a pressure vessel and passed under 800 to 2300 p.s.i. pressure through a spiral of copper tubing for temperature equilibration, then through the chromatographic column which is made of glass, enclosed in a pressure tube and easily removable. The apparatus is mounted in an insulating box lined with heating foil. A propeller circulates the air. Thermocouples are inserted into the entrance of the column. In one modification a high pressure gauge glass was substituted for part of the pressure tube allowing visual inspection during runs. After applying the porphyrins and charging the pressure vessel the apparatus is heated above the critical temperature. Then the gas flow is started and the pressure raised to the desired level. The rate of flow is determined at ambient pressure with a pneumatic trough at the outlet.

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