Microbiological 16-Oxidation of Estr-4-en-3-one

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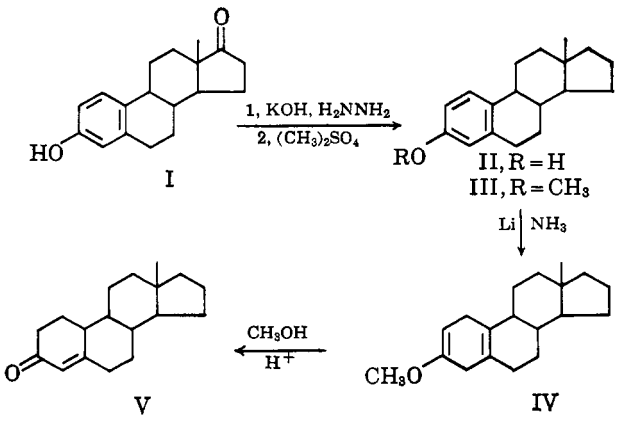
The preparation of estr-4-en-3-one and its use as a substrate in fermentations to yield 16-substituted products are described. Microbiological dehydrogenation of the products produced the known 3-hydroxyestra-1,3,5(10)-trien-16-one.

Microbiological transformation of the previously unreported steroid, estr-4-en-3-one (V), was attempted in order to provide intermediates for the preparation of various hydroxylated derivatives of 3-hydroxyestra-1,3,5(10)-triene (II). Direct fermentations of II with many microorganisms failed to provide such products in significant quantity.

The fermentation substrate, estr-4-en-3-one (V), was prepared by the route outlined in Scheme I. A Huang-

SCHEME I



Minlon reduction[1] of estrone (I) and methylation of the intermediate 3-hydroxyestra-1,3,5(10)-triene (II) produced 3-methoxyestra-1,3,5(10)-triene[2] (III) in good yield. A modified Birch reduction[3] of III yielded the previously unreported 3-methoxyestra-2,5(10)-diene (IV), which was readily hydrolyzed to V.

Three crystalline products (two monohydroxylated, VI and VIII, and a dione, VII) were isolated from a fermentation of estr-4-en-3-one (V) with *Bacillus megaterium* (NRRL-B938). The relative proportions of these products varied from run to run since the fermentation conditions and nutrient media also differed. Compounds VI and VII were also isolated from a fermentation of V with *Cephalosporium acremonium* (NRRL 3092). Since oxidation of both VI and VIII with chromic acid in acetone provided the dione VII, it was apparent that the substrate V had been oxidized at the same carbon atom to yield the three products, and also that the newly introduced hydroxyl groups in VI and VIII were epimeric. Moreover, D-ring substitution was inferred, since the infrared spectrum of VII showed the presence of a five-membered ring ketone. Since the products, VI, VII, and VIII were demonstrably different from the C-17 oxygenated compounds 19-nortestosterone and 19-norandrostenedione, oxidation could only have occurred at C-15 or C-16. Fermentations of VI, VII, and VIII with *Nocardia coralina* (ATCC 999) aromatized the A ring with concurrent oxidation of the hydroxyl groups of VI and VIII to provide in good yield the known[4] 3-hydroxyestra-1,3,5(10)-trien-16-one (IX), whose identity was confirmed by preparation of the benzoate (X). These results confirmed the position of substitution in compounds VI, VII, and VIII, but left unresolved the configuration of the hydroxyl groups in VI and VIII.

Our assignment of configuration is based on three considerations. Fermentation of V with *Streptomyces roseochromogenes* (ATCC 3347), a culture noted for 16α-hydroxylation of a wide range of steroids,[5] yielded primarily VIII. Secondly, the changes in molecular rotation on acetylation [$-54°$ for VIII and $+6°$ for VI (Table I)] agree with the $-37°$ for α and $+17°$ for β found for the analogous estrone derivatives.[6] Finally, reduction of the dione VII with aluminum *t*-butoxide[7] yielded mostly VI. From these data it appears that VI is 16β-hydroxyestr-4-en-3-one and VIII is 16α-hydroxyestr-4-en-3-one.

TABLE I

MOLECULAR ROTATION DIFFERENCES OF 16-SUBSTITUTED STEROIDS

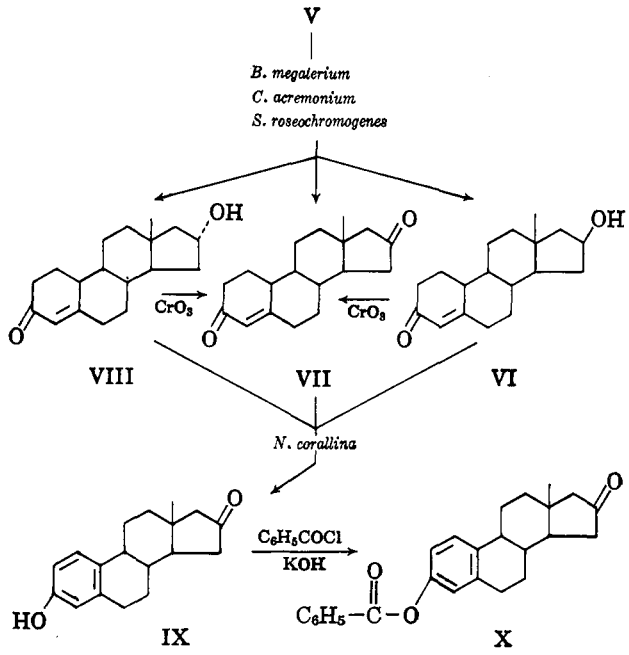
Steroid	MD, deg.	ΔMD, deg. (type)
Estr-4-en-3-one	+62	...
Estr-4-ene-3,16-dione	−400	−462 (C=O)
16α-Hydroxyestr-4-en-3-one	+54	−8 (α-OH)
16α-Acetoxyestr-4-en-3-one	±0	−54 (α-OH → α-OAc)
16β-Hydroxyestr-4-en-3-one	+62	±0 (β-OH)
16β-Acetoxyestr-4-en-3-one	+68	+6 (β-OH → β-OAc)
3-Hydroxyestra-1,3,5(10)-triene[a]	+236	...
3-Hydroxyestra-1,3,5(10)-trien-16-one[b]	−235	−471 (C=O)
3,16α-Dihydroxyestra-1,3,5(10)-triene[c]	+231	−5 (α-OH)
16α-Acetoxy-3-hydroxyestra-1,3,5(10)-triene[c,d]	+194	−37 (α-OH → α-OAc)
3,16β-Dihydroxyestra-1,3,5(10)-triene[c]	+202	−34 (β-OH)
16β-Acetoxy-3-hydroxyestra-1,3,5(10)-triene[c,d]	+219	+17 (β-OH → β-OAc)

[a] V. Prelog, L. Ruzicka, and P. Wieland, *Helv. Chim. Acta*, **28**, 250 (1945). [b] See ref. 1. [c] See ref. 6. [d] Calculated by subtracting the Δ^{OAc} value for estrone from the lit.[6] MD values for the 3,16-diacetates.

Microbiological 16β-hydroxylation of steroids has been only rarely reported. Herzog, *et al.*,[8] isolated

(1) Huang-Minlon, *J. Am. Chem. Soc.*, **71**, 3301 (1949).
(2) A. Butenandt and U. Westphal, *Z. physiol. Chem.*, **223**, 147 (1934).
(3) A. L. Wilds and A. J. Nelson, *J. Am. Chem. Soc.*, **75**, 5366 (1953).
(4) M. N. Huffman and M. H. Lott, *ibid.*, **73**, 878 (1951).
(5) C. E. Holmlund, L. I. Feldman, R. H. Blank, N. Barbacci, and B. Nielsen, *Sci. Rept. Inst. Super. Sanita*, **1**, 289 (1961).
(6) M. N. Huffman and M. H. Lott, *J. Biol. Chem.*, **215**, 627 (1955).
(7) J. W. Ralls, U. S. Patent 2,778,841 (1957).
(8) H. L. Herzog, M. J. Gentles, A. Basch, W. Coscarelli, M. E. A. Zeitz, and W. W. Charney, *J. Org. Chem.*, **25**, 2177 (1960).

SCHEME II



nine crystalline products from a fermentation of testosterone with *Wojnowicia graminis*. The major product was 16α-hydroxytestosterone; 16β-hydroxytestosterone and 16-ketotestosterone were isolated in much smaller amounts. J. de Flines, *et al.*,[9] found that 16β-hydroxy-19-nortestosterone and, to a considerably lesser extent, 16-keto-19-nortestosterone were produced in fermentations of 19-nortestosterone with *Mycosphaerella latebrosa*. Dodson and Mizuba,[10] who obtained 16β-hydroxytestosterone and 16-ketotestosterone from the fermentation of androstenedione with *Corticium centrifugum*, have suggested that the 16β-hydroxylated product might have resulted from 16α-hydroxylation of androstenedione, followed by isomerization of this 16,17-ketol, and reduction of the resulting 16-keto group.

Laboratory-scale fermentations of V, VI, VII, and VIII with the three organisms, *C. acremonium*, *B. megaterium*, and *S. roseochromogenes*, all resulted in mixtures of VI, VII, and VIII. These results show that a mechanism exists for the interconversion of 16α- and 16β-isomers, probably *via* the 16-ketone, and suggest the presence of 16α-hydroxy steroid and 16β-hydroxy steroid dehydrogenases in these fermentations. In fermentations of V with *C. acremonium* and *B. megaterium*, 16β-hydroxyestr-4-en-3-one (VI) appeared to be formed first, probably by 16β-hydroxylation of V, and subsequently to be converted to VII and VIII. With *S. roseochromogenes*, however, the first abundant product was 16α-hydroxyestr-4-en-3-one (VIII); VI and VII were formed later.

C. acremonium has been reported to form testololactone and testolic acid from androstenedione and progesterone.[11] No analogous product was found in the fermentation of VII with this culture. Apparently the enzyme(s) that oxidize(s) the 17- or 20-keto steroids is unable to catalyze a similar reaction with the 16-keto steroid.

(9) J. de Flines, W. F. van der Waard, W. J. Mijs, and S. A. Szpilfogel, *Rec. trav. chim.*, **82**, 121 (1963).

(10) R. M. Dodson and S. Mizuba, *J. Org. Chem.*, **27**, 698 (1962).

(11) C. E. Holmlund, R. H. Blank, K. J. Sax, and R. H. Evans, Jr., *Arch. Biochem. Biophys.*, **103**, 105 (1963).

Experimental

Melting points are corrected. Partition chromatography was performed on columns of diatomaceous earth moistened with 50% (w./v.) of the lower phase of a solvent system composed of water, methanol, dioxane, and cyclohexane. The particular solvent system employed is identified by the respective volume ratios of these individual components, *e.g.*, 2:4:1:10. The columns were eluted with the upper phase of the same solvent system or with the upper phase of systems with different volume ratios. Adsorption chromatography was done on columns of silica gel (Davison, Grade 62, 60-200 mesh). Fractions from both partition and adsorption columns are identified by reference to the volume of eluate collected. Thin-layer chromatography was done on silica gel G[12] activated for 30 min. at 100°. The plates were developed with benzene–acetone (7:3 by volume), and the steroids were detected by spraying the plates with sulfuric acid and heating until the steroid was charred.

3-Methoxyestra-1,3,5(10)-triene (III).—Crude damp 3-hydroxyestra-1,3,5(10)-triene (II), prepared from 99.5 g. of estrone by Huang-Minlon reduction,[1] was dissolved in a mixture of 1.6 l. of dioxane and 1 l. of water, and the solution was treated with 33 g. of 86% potassium hydroxide in 150 ml. of water. Dimethyl sulfate (45 ml.) was added, and the mixture was heated on the steam bath for 2 hr. During this time additional dioxane and water were added to prevent the separation of a second phase. An additional 33 g. of potassium hydroxide and 20 ml. of dimethyl sulfate were added and heating was continued for 2 hr. The reaction mixture was poured onto 2 kg. of ice, and the crude 3-methoxyestra-1,3,5(10)-triene (III) was collected and washed with water. On fractional crystallization from methanol a total of 86.5 g. (86.5% based on estrone) of III, m.p. 78–79°, was obtained. Butenandt and Westphal[2] reported m.p. 76.5°.

3-Methoxyestra-2,5(10)-diene (IV).—A mixture of 7.5 g. of 3-methoxyestra-1,3,5(10)-triene in 20 ml. of ethanol, 100 ml. of anhydrous ethyl ether, and 500 ml. of liquid ammonia was treated with 7 g. of lithium metal, which was added in small pieces during stirring over a 2-hr. period. The reaction was continued until an aliquot diluted with ethanol no longer showed appreciable absorption at 280 mμ. After evaporation of the ammonia, the excess lithium was destroyed by careful addition of ethanol and water. The mixture was diluted with water and the product was extracted with ether. After washing and drying, the ether extract was concentrated and the product was crystallized. Fractional crystallization from ether gave a first crop of 4.8 g. of IV, m.p. 91–93°, $[\alpha]^{25}D$ +69° ($CHCl_3$).

Estr-4-en-3-one (V).—A suspension of 5.7 g. of 3-methoxyestra-2,5(10)-diene in 48 ml. of methanol and 2 ml. of concentrated hydrochloric acid was warmed gently on a steam bath until solution was complete. After about 15 min. the solution was treated with 5 ml. of 5 *N* sodium hydroxide solution and a little water. The mixture was cooled in a Dry Ice–methanol bath, and 4.2 g. of crystalline estr-4-en-3-one, m.p. 63.4–65°, was collected. Recrystallization of this material from aqueous methanol yielded pure V, m.p. 65.5–66.75°, $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,000), $[\alpha]^{25}D$ +44° ($CHCl_3$), $[\alpha]^{25}D$ +24 (CH_3OH).

Anal. Calcd. for $C_{18}H_{26}O$ (mol. wt. 258.39): C, 83.66; H, 10.14. Found: C, 83.97; H, 10.32.

Isolation of 16β-Hydroxyestr-4-en-3-one (VI) from the Fermentation of Estr-4-en-3-one (V) with *B. megaterium*.—Thirty liters of a medium consisting of 1500 g. of glucose, 600 g. of lactalbumin hydrolysate,[13] and 150 g. of corn steep liquor was adjusted to pH 6.5 with dilute sodium hydroxide solution, and was inoculated with 3.3% by volume of a 24-hr. inoculum of *B. megaterium*. The fermentation mash was stirred and aerated at 28° during 48 hr., at which time a solution of 3.0 g. of estr-4-en-3-one in 100 ml. of methanol was added. After an additional 6.5 hr. the fermentation mash was adjusted to pH 6.5 with dilute hydrochloric acid, and was extracted with an equal volume of chloroform. The chloroform extract was separated, and the aqueous layer was twice extracted with half its volume of fresh chloroform. The combined chloroform extract was concentrated under vacuum to 4 l., dried over magnesium sulfate, stirred with 5 g. of decolorizing carbon, and filtered. Concentration of the filtrate yielded an oily residue that was dissolved in 200 ml. of a 3:1 (v./v.) methylene chloride–hexane mixture. Adsorption chromatography on a 300-g. column on silica gel was carried out by elution.

(12) Obtained from E. Merck AG, Darmstadt; *cf.* E. Stahl, *Pharm. Rundschau*, **1**, 1 (1959).

(13) Sheffield Chemical, Norwich, N. Y.

with 1800 ml. of the above solvent mixture, followed by 8 l. of 2% acetone in methylene chloride, and 3 l. of 10% acetone in methylene chloride. The steroid obtained by evaporation of solvent from the 3.8–6.8-l. portion of the eluate was crystallized from acetone–hexane to yield 50 mg. of 16β-hydroxyestr-4-en-3-one (VI), m.p. 149–150.5°, $[\alpha]^{25}D$ +22.8 (*c* 1.05, CH_3OH), $\lambda_{max}^{CH_3OH}$ 239 mμ (ε 17,350).

Anal. Calcd. for $C_{18}H_{26}O_2$ (mol. wt. 274.39): C, 78.78; H, 9.55. Found: C, 79.21; H, 9.81.

16α-Hydroxyestr-4-en-3-one (VIII), 16β-Hydroxyestr-4-en-3-one (VI), and Estr-4-ene-3,16-dione (VII).—The fermentation of a second portion of estr-4-en-3-one (V, 2.0 g.) with *B. megaterium* was done in a medium composed of 400 g. of lactalbumin hydrolysate,[13] 400 g. of glucose, and 144 g. of corn steep liquor in water to make 20 l. Five per cent by volume of a 24-hr. inoculum was added and the culture was grown for 24 hr. prior to the addition of the steroid in methanol solution. After 23 hr. the fermentation was stopped, and the steroid was extracted with chloroform as described above. Evaporation of the chloroform extract afforded 133 g. of viscous oil. Chromatography on a 600-g. column of silica gel was accomplished by elution with 50% methylene chloride in hexane (1.2 l.), 60% methylene chloride in hexane (2 l.), 80% methylene chloride in hexane (2 l.), methylene chloride (7 l.), 5% ether in methylene chloride (1 l.), 10% ether in methylene chloride (1 l.), 50% ether in methylene chloride (4 l.), and 5 l. of ether.

Partition chromatography of the residue obtained on evaporation of solvent from the 8.0–11.7-l. portion of the eluate was carried out on a 400-g. column of diatomaceous earth moistened with 200 ml. of the lower phase of a 2:4:1:10 solvent system. Elution of the column with 1 l. of the upper phase of the above system and 2 l. of the upper phase of a 2:6:1:10 system provided a fraction (1.0–1.9 l. of eluate) which yielded, on concentration and crystallization of the residue from aqueous methanol, 700 mg. of estr-4-ene-3,16-dione (VII), m.p. 139.5–140.5°. Additional product was recovered from the mother liquors.

The 11.7–15.9-l. fraction collected from the adsorption column was concentrated to a residue, which was chromatographed on a partition column of 75 g. of diatomaceous earth moistened with 37.5 ml. of the lower phase of a 2:2:2:10 solvent system. Elution of the column with the upper phase of this system produced two steroid-containing fractions, 90–165 ml. and 215–330 ml. Concentration of the first fraction and crystallization of the residue from aqueous methanol yielded 70 mg. of estr-4-ene-3,16-dione, m.p. 135–136.5°. Similar work-up of the second fraction gave 13 mg. of 16β-hydroxyestr-4-en-3-one (VI), m.p. 146.8–148.3°.

The 17.5–18.7-l. fraction collected from the adsorption column was concentrated to a residue which was further purified by partition chromatography on a column of 300 g. of diatomaceous earth moistened with 150 ml. of the lower phase of a 2:2:4:10 solvent system. Elution with the upper phase of this solvent system and concentration of the 825–1265-ml. fraction yielded a residue containing 16α-hydroxyestr-4-en-3-one (VIII), which was crystallized from ethyl acetate–hexane. Recrystallization of the product from the same solvents yielded 157 mg. of VIII, m.p. 163–164°, $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,600), $[\alpha]^{25}D$ +21° (CH_3OH).

Estr-4-ene-3,16-dione (VII). A.—A solution of 50 mg. of 16β-hydroxyestr-4-en-3-one (VI) in 25 ml. of chromic acid–acetone reagent[14] was allowed to stand at room temperature for 10 min. Methanol was added to consume excess oxidant, and the solution was diluted with water and extracted with methylene chloride. The extract was dried over magnesium sulfate, filtered, and the solvent was evaporated. Crystallization of the residue from aqueous methanol and aqueous acetone afforded 22 mg. of estr-4-ene-3,16-dione (VII), m.p. 138.5–139.5°, $[\alpha]^{25}D$ −147° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 17,400); λ_{max}^{KBr} 5.73 (5-membered ring ketone), 5.99, and 6.17 μ.

B.—A solution of 60 mg. of 16α-hydroxyestr-4-en-3-one (VIII) in 30 ml. of chromic acid–acetone reagent[14] was treated as above. The crude product was purified by partition chromatography on a 20-g. diatomaceous earth column using a 2:4:1:10 solvent system. The 41–90-ml. fraction was concentrated to a residue, which was crystallized from aqueous methanol; 32 mg. of estr-4-ene-3,16-dione (VII), m.p. 140.5–141.5°, $\lambda_{max}^{CH_3OH}$ 239 mμ (ε 16,300), $[\alpha]^{25}D$ −147° (CH_3OH), was collected.

(14) One milliliter of a stock solution of 20 g. of chromic anhydride and 32 g. of concentrated sulfuric acid in water to make 100 ml. is diluted to 100 ml. with acetone; *cf.* S. C. Pan, *Anal. Chem.*, **34**, 766 (1962).

Anal. Calcd. for $C_{18}H_{24}O_2$ (mol. wt. 272.37): C, 79.37; H, 8.88. Found: C, 79.51; H, 9.05.

Isolation of Estr-4-ene-3,16-dione (VII) and 16β-Hydroxyestr-4-en-3-one (VI) from the Fermentation of Estr-4-en-3-one (V) with *Cephalosporium acremonium*.—An inoculum prepared by growing a culture of *Cephalosporium acremonium* (NRRL 3092) as described under Laboratory Fermentation Studies was used to inoculate three 750-ml. portions of the same medium in 4-l. erlenmeyer flasks. Each flask was shaken for 24 hr. at 28°, and a concentrated solution of 500 mg. of V in methanol was divided equally among the three vessels. After shaking at 28° for 51 hr. the fermentations were combined, and the pH adjusted to 2.5 with 5 *N* hydrochloric acid. The mixture was extracted twice with an equal volume of methylene chloride. After filtration of the aqueous phase, the filtrate was again extracted with methylene chloride and the combined extract was concentrated to an oily residue (8.5 g.). Partition chromatography of the residue on a 150-g. column of diatomaceous earth moistened with 75 ml. of the lower phase of a 2:4:1:10 solvent system was accomplished by elution with 500 ml. of the upper phase of the above system, 700 ml. of a 2:2:1:10 system, and 1 l. of a 2:2:2:10 system. Crystallization from acetone–water of the residue obtained on evaporation of the 420–630-ml. fraction gave 10 mg. of estr-4-ene-3,16-dione (VII), m.p. 133–137°, which was identified by its infrared absorption spectrum and by mixture melting point with an authentic sample. Crystallization from acetone–hexane of the residue obtained on evaporation of the eluate between 1.2 and 1.6 l. gave 13 mg. of 16β-hydroxyestr-4-en-3-one (VI), m.p. 146.5–148.5°, which was similarly identified.

Isolation of 16α-Hydroxyestr-4-en-3-one (VIII) from the Fermentation of Estr-4-en-3-one (V) with *S. roseochromogenes* (ATCC 3347).—Five per cent of a 48-hr. inoculum of *S. roseochromogenes* was used to inoculate 20 l. of a growth medium composed of 4% corn starch, 2.5% corn steep liquor, 0.5% calcium carbonate, 0.5% dibasic potassium phosphate, and 0.2% lard oil in water. After the culture was grown for 24 hr. at 28° a solution of 2 g. of V in 100 ml. of methanol was added to the fermentor, and the fermentation was continued for 30 hr. The mash was extracted once with an equal volume of chloroform and re-extracted twice with half-volumes of chloroform. The combined extract was concentrated to an oil, which was subjected to partition chromatography on a column composed of 700 g. of diatomaceous earth moistened with 350 ml. of the lower phase of a 2:4:1:10 solvent system. The column was eluted with 1.2 l. of the upper phase of this system and with the upper phase of 2:4:2:10 (2.5 l.), 2:2:2:10 (2.5 l.), 2:2:4:10 (3.4 l.), and 2:2:10:10 systems. The steroid obtained on concentration of the 6.0–10.0-l. fraction was fractionally crystallized from ethyl acetate–hexane to yield 280 mg. of VIII, m.p. 163–163.5°, $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,200), $[\alpha]^{25}D$ +20° (CH_3OH).

Anal. Calcd. for $C_{18}H_{26}O_2$ (mol. wt. 274.39): C, 78.78; H, 9.55. Found: C, 78.93, 78.41; H, 9.78, 9.71.

Isolation of 3-Hydroxyestra-1,3,5(10)-trien-16-one (IX) from the Fermentation of Estr-4-ene-3,16-dione with *Nocardia corallina* (ATCC 999).—Three 4-l. erlenmeyer flasks each containing 750 ml. of a growth medium composed of 10 g. of glucose, 1 g. of yeast extract (Difco), 2.5 g. of sodium chloride, 4 g. of beef extract (Armour), and 4 g. of peptone (Difco) per liter of water were inoculated with 100 ml. of a culture of *Nocardia corallina* grown at 37° in the same medium for 6 hr. and the flasks were incubated at 24° on a reciprocating shaker for 16 hr. Under aseptic conditions 165 mg. of estr-4-ene-3,16-dione was added to each flask in 35 ml. of methanol, and the fermentation was continued for 24 hr. The combined fermentation mixture was stirred with an equal volume of methylene chloride. After separation of the phases, the aqueous phase was re-extracted with two half-volumes of methylene chloride. Evaporation of the solvent from the combined extract left an oily residue which was chromatographed on a 200-g. column of diatomaceous earth moistened with 100 ml. of a 2:4:1:10 solvent system. The column was eluted with 900 ml. of upper phase from the above system, 900 ml. of a 2:2:1:10 system, and 1 l. of a 2:2:2:10 system. The solvent was evaporated from the 686–3411-ml. fraction, and the residue was dissolved in ethyl acetate. The solution was treated with decolorizing carbon, filtered, and concentrated with hexane to yield 333 mg. (67%) of 3-hydroxyestra-1,3,5(10)-trien-16-one, m.p. 244.5–246°. Huffman and Lott[4] report m.p. 243.5–245.5 for this product.

3-Benzoyloxyestra-1,3,5(10)-trien-16-one (X).—One drop of benzoyl chloride was added to a solution of 10 mg. of 3-hydroxy-

estra-1,3,5(10)-trien-16-one in 0.4 ml. of 20% sodium hydroxide, and the solution was mixed. When the product crystallized, the solution was diluted with water and filtered, and the product was recrystallized three times from methanol. The 5 mg. of X thus obtained melted at 225.5–229°. Huffman and Lott[4] report m.p. 223.5–224.5°.

16β-Acetoxyestr-4-en-3-one.—Acetylation of 16β-hydroxyestr-4-en-3-one (75 mg.) in 2 ml. of pyridine with 0.5 ml. of acetic anhydride yielded 75 mg. of crude product, m.p. 87.5–89.5°. Two recrystallizations from aqueous acetone yielded 35 mg. of 16β-acetoxyestr-4-en-3-one, m.p. 90–90.5°, $[\alpha]^{25}D$ +21.5° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 15,000).

Anal. Calcd. for $C_{20}H_{28}O_3$ (mol. wt. 316.42): C, 75.91; H, 8.92. Found: C, 75.67; H, 9.01.

16α-Acetoxyestr-4-en-3-one.—Acetylation of 16α-hydroxyestr-4-en-3-one (100 mg.) in 5 ml. of pyridine with 1 ml. of acetic anhydride yielded an oil, which showed no optical rotation, $\lambda_{max}^{CH_3OH}$ 239.5 mμ (ε 15,000). The infrared absorption spectrum showed the presence of an ester group but no hydroxyl group; λ_{max}^{KBr} 5.72, 5.93, 6.12, and 7.99 μ.

Aluminum *t*-Butoxide Reduction of Estr-4-ene-3,16-dione (VII).—A solution of 100 mg. of VII in 10 ml. of benzene and 1 ml. of 2-butanol was slowly distilled until 2.5 ml. of distillate was collected. Then 74 mg. of aluminum *t*-butoxide was added, and the mixture was refluxed for 5 hr. After cooling and dilution with 10 ml. of benzene the mixture was washed once with 30 ml. of 5% sodium hydroxide solution and thrice with 10-ml. portions of water. The organic layer was dried over magnesium sulfate, and the residue obtained on evaporation of solvent was chromatographed on a 25-g. silica gel column by development with 425 ml. of methylene chloride–hexane (1:1), 500 ml. of methylene chloride–hexane (3:1), 1 l. of methylene chloride, and 500 ml. of ether–methylene chloride (1:9).

Examination of the eluate by paper chromatography showed starting material in the 490–970-ml. fraction. The 1480–1820-ml. fraction contained 16β-hydroxyestr-4-en-3-one, and the 2170–2330-ml. fraction contained a smaller amount of the 16α-isomer.

Laboratory Fermentation Studies.—Laboratory-scale fermentations were conducted in the following manner. Inoculum was prepared by transferring an aqueous suspension of the culture to a 500-ml. erlenmeyer flask containing 100 ml. of a medium consisting of lactalbumin hydrolysate[13] (20 g.), glucose (20 g.), corn steep liquor (5 g.), and water to make 1000 ml. The pH was adjusted to 7.0. The culture was incubated on a reciprocating shaker; *B. megaterium* was incubated at 37° for 7 hr.; *C. acremonium* and *S. roseochromogenes* were incubated at 28° for 64 hr. In each instance 5 ml. of the microbial suspension was used to inoculate 100 ml. of fresh medium in 500-ml. erlenmeyer flasks. All cultures were than shaken at 28° for 24 hr. prior to addition of the steroid substrate. Each steroid was added in methanolic solution to provide a concentration of 100 μg./ml. in the flask. Aliquots were removed at intervals and extracted with a three-volume portion of ethyl acetate, and the extract was analyzed by thin layer chromatography.

A mixture of compounds, VI, VII, and VIII, was observed in one or more of the samples removed from the fermentations of V, VI, VII, and VIII with each organism. The mobilities of these compounds on silica gel G developed with benzene–acetone (7:3 v./v.) were as follows: V, R_f 0.68–0.74; VI, R_f 0.42–0.46; VII, R_f 0.55–0.62; and VIII, R_f 0.34–0.38. After spraying with sulfuric acid and heating, the 3,16-dione (VII) shows a distinctive olive green color prior to charring.

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Application of Spin Decoupling and 100-Megacycle Spectra to Characterization of Carbohydrates. Novel Synthesis of a Cyclohexanetetrol[1,2]

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Reaction of 5,6-anhydro-*allo*-inositol diketal with hydrogen bromide–acetic acid unexpectedly gave a dibromocyclohexanetetrol tetraacetate, from which was obtained a new cyclohexanetetrol, m.p. 193°. By use of spin decoupling and 100-Mc. spectra, this tetrol was shown to have the structure 1,2,3,5 and the configuration *meso* (123/5). Another diastereomer, m.p. 180°, prepared in 1954 from *myo*-inositol, was shown by 60- and 100-Mc. spectra to have the configuration *meso* (13/25). Configurational assignments have also been made to two optically active diastereomers, prepared by Dangschat and Fischer in 1939. By means of rotation predictions, it is now shown that their isomers of reported molecular rotations −90 and −12° have the absolute configurations L (12/35) and L (125/3), respectively.

For n.m.r. configurational studies, spin coupling is a generally useful and often indispensable phenomenon, but there are times when it produces such spectral complexity that interpretation becomes impossibly difficult. This has been especially true for carbohydrate spectra,[5] because of the numerous similar functional groups.

It now appears that such difficulties can largely be overcome, owing to two lately reported n.m.r. developments. The first development, spin decoupling,[6,7] permits the simple uncoupled signal of almost any chosen proton, or set of protons, in a molecule to be observed. Although anticipated by Bloch[6] as early as 1954, spin decoupling has only recently become fully practical and convenient for general use. The

(1) Presented by G. E. M. to the Division of Organic Chemistry at the 144th National Meeting of the American Chemical Society, Los Angeles, Calif., April, 1963.

(2) Paper 18 on Cyclitol Stereochemistry by G. E. McCasland and coworkers; for recent papers in this series, see *J. Org. Chem.*, **28**, 2096 (1963); **29**, 724 (1964); *J. Am. Chem. Soc.*, **85**, 2866 (1963).

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(5) For our own previous n.m.r. studies on carbohydrates (cyclitols), see G. E. McCasland, S. Furuta, L. F. Johnson, and J. N. Shoolery: (a) *J. Org. Chem.*, **28**, 894 (1963); (b) *J. Am. Chem. Soc.*, **83**, 4243 (1961); (c) **83**, 2335 (1961); (d) (with A. Furst), *J. Org. Chem.*, **28**, 456 (1963); (e) G. E. McCasland, S. Furuta, and A. Furst, *ibid.*, **29**, 724 (1964).

(6) Double irradiation was originated by F. Bloch [*Phys. Rev.*, **93**, 944 (1954); **94**, 496 (1954)]. The theory was developed by A. Bloom and J. N. Shoolery [*ibid.*, **97**, 1261 (1955)]. The first application to proton-proton spin decoupling was by W. Anderson [*ibid.*, **102**, 151 (1956)], who studied 2,3-dibromopropene and 2,2-dichloroacetaldehyde. For a recent review, see J. Baldeschwieler and E. Randall, *Chem. Rev.*, **63**, 81 (1963).

(7) The spin-decoupling method used here is that described by R. Freeman and D. Whiffen [*Mol. Phys.*, **4**, 321 (1961)]. The instrumentation is described by L. F. Johnson, Varian Associates Technical Information Bulletin, Vol. 3, No. 3, Palo Alto, Calif., 1963, pp. 5–7, 11–13.