TABLE IV

2,2′-BIPYRROLES (IV)

IV	M.p., °C.	Yield, %	Ultraviolet absorption[a] λ_{max} (ϵ)	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
a (= b)	134–135	29	289 (16,000), 283 (16,400), 278 (15,600)	$C_9H_{10}N_2$	73.9	73.9	6.9	7.0	19.2	19.1
c	190–191	24	295 (14,700), 290 (14,900), 280 (12,800)	$C_{10}H_{12}N_2$	75.0	75.3	7.6	7.7	17.5	17.3
d	200–201	22	288 (16,900), 281 (17,000), 276 (16,200)	$C_9H_{10}N_2$	73.9	74.3	6.9	7.2	19.2	19.2
e	197–198	25	288 (16,600), 281 (16,900), 276 (16,200)	$C_9H_{10}N_2$	73.9	74.1	6.9	7.0	19.2	19.1
f	156–157	46	267 (16,500), 231 (20,600)	$C_{12}H_{14}N_2O_2$	66.0	66.0	6.5	6.4	12.8	13.2
g	231–232	79	325 (26,900), 220 (13,600)	$C_{10}H_{10}N_2O_2$	63.2	63.5	5.3	5.5	14.7	14.5
h	95–96	45	314 (16,800)	$C_{11}H_{12}N_2O_2$	64.7	64.9	5.9	6.0	13.7	14.1

[a] Ultraviolet spectra were measured in ethanol.

with aqueous potassium carbonate, evaporating the ether, and subliming (110° at 0.1 mm.) the residue. **Methyl 3,4-dimethylpyrrole-2-carboxylate** resulted in quantitative yield, m.p. 103–105°; ultraviolet absorption: λ_{max} 285 mμ (ϵ 25,000).

Anal. Calcd. for $C_8H_{11}NO_2$: C, 62.7; H, 7.2; N, 9.2. Found: C, 62.8; H, 7.3; N, 9.0.

2,2′-(1′-Pyrrolinyl)pyrroles.—Condensation between a pyrrole and a 2-pyrrolidinone was carried out in all cases by the following general procedure.

To a solution of 8.0 g. (0.1 mole) of 2-methylpyrrole and 4.0 g. (47 mmoles) of 2-pyrrolidinone in 30 ml. of ethylene dichloride at 0° and in a nitrogen atmosphere, was added 12.0 g. (79 mmoles) of phosphorus oxychloride over a 1-hr. period. The reaction mixture was stirred at room temperature for 2 hr., and the product was isolated as described.[5a] The resulting pyrrolinylpyrrole was recrystallized from hexane–benzene and sublimed at 100° at 0.1 mm.

5,5′-Dimethyl-2,2′-(1′-pyrrolinyl)pyrrole (IIIc) required chromatography on neutral alumina for purification and was eluted with benzene–chloroform, 1:1.

3-Methyl-2,2′-(1′-pyrrolinyl)pyrrole (IIIe) was separated from the 4-methyl isomer (IIId) by continuous chromatography on neutral alumina using the apparatus shown in Fig. 1. After 10 days' continuous elution with benzene, all the 3-methyl isomer (IIIe) had been removed, and the 4-methyl isomer (IIId), present in equal amount, was removed rapidly with benzene–chloroform, 1:1.

2,2′-Bipyrroles.—Catalytic dehydrogenation of the 2,2′-(1′-pyrrolinyl)pyrroles to give the corresponding 2,2′-bipyrroles was carried out in all cases by the following general procedure.

A mixture of 2.4 g. (16 mmoles) of 5-methyl-2,2′-(1′-pyrrolinyl) pyrrole, 2.5 g. of 30% palladium on carbon, and 225 ml. of di-*n*-hexyl ether was heated at 200° for 2 hr. using a nitrogen sweep. The hot solution was filtered, 500 ml. of hexane was added to the filtrate, and the solution was now cooled at −70° for 24 hr. The resulting crystals were removed by filtration and sublimed at 120° at 0.1 mm. to give a 29% yield of 5-methyl-2,2′-bipyrrole (IVa), m.p. 134–135°.

Material identical with the 5-methyl-2,2′-bipyrrole (IVa) prepared by catalytic dehydrogenation was obtained when 5-methoxycarbonyl-2,2′-bipyrrole (IIIg, 0.53 g., 2.8 mmoles) in 60 ml. of tetrahydrofuran was added slowly to 5.8 ml. of 1 *M* lithium aluminum hydride in tetrahydrofuran and heated under reflux for 3 hr. Cooling, addition of ice, filtering, extracting with chloroform, evaporating the chloroform, and subliming gave 2.8 g. (67% yield) of IVa.

Steroids. CCLXI.[1] Microbiological Hydroxylation of Estrane Derivatives with *Fusarium moniliforme*

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Incubation of estrone (Ia) with *Fusarium moniliforme* afforded 15α-hydroxyestrone (Ib). Incubation of estradiol (IIa) with the same microorganism provided 15α-hydroxyestradiol (IIc), while 6β-hydroxyestradiol 3-methyl ether (Vb) was obtained by microbial incubation of estradiol 3-methyl ether (IIb). The structure determination of these substances, obtained by enzymatic transformations, results from both chemical and nuclear magnetic resonance evidence.

It has been known for several years that some microorganisms produce enzymes capable of stereospecifically hydroxylating the steroid molecule at a definite position.[2,3] While microbiological hydroxylation of androstane and pregnane molecules has been extensively investigated, little has been reported so far on microbial transformations of estrane derivatives.[3,4]

The present study reports the elucidation of the structure of the compounds obtained by incubation of estrone (Ia), estradiol (IIa), and estradiol 3-methyl ether (IIb) with *Fusarium moniliforme*. This microorganism, corresponding to an imperfect stage of *Gibberella fujikuroi*,[5] is known to hydroxylate the androstane and pregnane molecules at C-6β and/or C-15α.[6] However, no work on the fermentation of estrane derivatives with this microorganism has been reported.

Incubation of estrone (Ia) with *Fusarium moniliforme*[7] provided a crystalline substance (Ib), the elemental analysis of which indicated the introduction of one hydroxyl group into the estrone molecule. While

(1) Part CCLX: A. D. Cross and P. W. Landis, *J. Am. Chem. Soc.*, in press.

(2) Several review articles on this subject have appeared recently, for example: (a) E. Vischer and A. Wettstein, "Advances in Enzymology," F. F. Nord, Ed., Interscience Publishers, Inc., New York, N. Y., 1958, p. 237; (b) L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p. 672; (c) L. M. Kogan, *Russ. Chem. Rev.*, **31**, 294 (1962); (d) M. Shirasaka, *Ann. Sankyo Res. Lab.*, **15**, 1 (1963).

(3) C. Tamm, *Angew. Chem.*, **74**, 225 (1962).

(4) A. I. Laskin, J. Fried, P. Grabowich, B. Junta, and C. D. Meyers, *Bact. Proc.* 106 (1963).

(5) See, for example, W. C. Snyder and H. N. Hansen, *Am. J. Botany*, **32**, 657 (1945).

(6) See (a) A. Čapek and O. Hanč, *Folia Microbiol.* (Prague), **5**, 251 (1960); (b) B. Klüger, *et al.*, *Naturwiss.* **44**, 40 (1957); (c) P. D. Meister, *et al.*, unpublished work cited in S. H. Eppstein, *et al.*, *Vitamines and Hormones*, **14**, 359 (1956); (d) see also ref. 2a.

(7) See Experimental. Further microbiological aspects of these incubations as well as other related experiments will be published elsewhere.

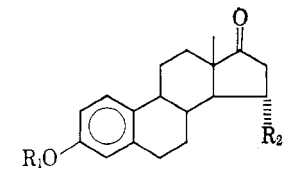
acetylation of Ib with acetic anhydride in pyridine solution at room temperature afforded a diacetoxy derivative (Ic), benzoylation of the diol (Ib), under similar conditions, gave a monobenzoate (Id). The ultraviolet spectrum of this benzoate (Id) clearly showed the ester group to be at C-3, thus indicating the new hydroxyl group in Ib to be secondary but somewhat sterically hindered. Furthermore, the keto benzoate (Id) showed a negative ferric chloride test and its ultraviolet absorption spectrum was unchanged in alkaline medium indicating that the newly introduced hydroxyl was probably not phenolic. This point was confirmed when it was found that methylation of the diol (Ib) furnished a monomethyl ether (Ie). In the nuclear magnetic resonance (n.m.r.) spectrum of the ether (Ie), the methoxy function appears at 226 c.p.s. as a three-proton singlet and resonance for the aromatic C-1,C-2, and C-4 protons is clearly observed (see Experimental).

The keto diol (Ib) differed from the known compounds.[8-13]

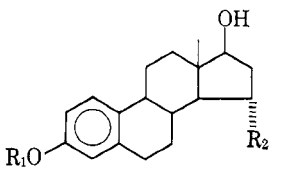
Sodium borohydride reduction of the ether (Ie) provided a diol (IId), which was also different from all known 3-methoxyestra-1,3,5(10)-trienediols.

Numerous n.m.r. studies have established[14] that 1,3-diaxial interactions of steroid angular methyl groups with substituents elsewhere in the molecule lead to pronounced downfield shifts of the methyl proton resonances. Careful examination of the available n.m.r. information clearly indicates that no such 1,3-interaction exists in either the diacetate (Ic), the monobenzoate (Id), or the methyl ether (Ie), thus excluding the 15β-configuration for the newly introduced hydroxyl group in Ib and its derivatives (Ic–e).

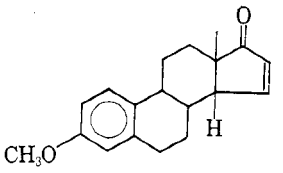
Among the various possibilities still available for the location of the hydroxyl group in the ketodiol Ib, it was felt, on the basis of previous microbiological incubation experiments conducted with this microorganism in other series,[6] that position 15α was the most probable. In order to verify this assumption, the incubated compound (Ib) was methylated to Ie and this substance was then dehydrated with phosphorus oxychloride in pyridine solution at room temperature.[15] Under these conditions, there was obtained a compound (III), showing a characteristic ultraviolet absorption spectrum typical of the newly introduced α,β-unsaturated cyclopentenone chromophore.[16]



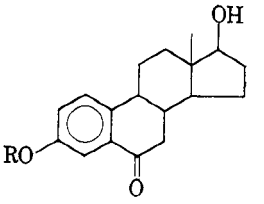
Ia, $R_1 = R_2 = H$
b, $R_1 = H$; $R_2 = OH$
c, $R_1 = C(=O)-CH_3$; $R_2 = O-C(=O)-CH_3$
d, $R_1 = C(=O)-C_6H_5$; $R_2 = OH$
e, $R_1 = CH_3$; $R_2 = OH$



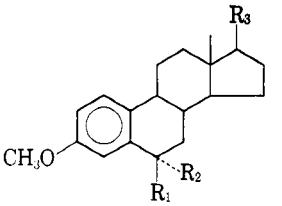
IIa, $R_1 = R_2 = H$
b, $R_1 = CH_3$; $R_2 = H$
c, $R_1 = H$; $R_2 = OH$
d, $R_1 = CH_3$; $R_2 = OH$



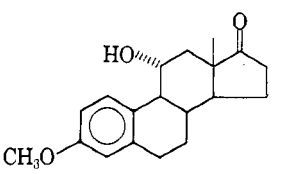
III



IVa, R = H
b, R = CH_3



Va, $R_1 = H$; $R_2 = R_3 = OH$
b, $R_1 = OH$; $R_2 = H$; $R_3 = OH$
c, $R_1 = R_3 = OAc$; $R_2 = H$



VI

The correctness of the assumption that the newly introduced hydroxyl group of Ib indeed had the 15α-configuration was further confirmed when this substance (Ib) was reduced with sodium borohydride to afford estra-1,3,5(10)-triene-3,15α,17β-triol (IIc). This compound was shown to be identical with an authentic sample of 15α-hydroxyestradiol (IIc) recently obtained[4,17] by fermentation of estradiol (IIa) with *Glomerella fusarioides*, *Glomerella glycines*, or *Aspergillus carneus*.

The same triol (IIe) was obtained by microbiological incubation of estradiol (IIa) with *Fusarium moniliforme*.

Incubation of estradiol 3-methyl ether (IIb) also gave a hydroxylated substance. This compound (Vb) was different from 15α-hydroxyestradiol 3-methyl ether (IId) (*vide supra*). Further insight into the structure and the stereochemistry of this metabolite (Vb) was gained by careful examination of the n.m.r. spectrum of the corresponding diacetate (Vc).

It is known from recent work[18] that the 6β-proton, but not the 6α-, is significantly coupled with the 4-olefinic proton. Moreover, it has been shown that for

(8) 6-Hydroxyestrones: (a) R. Y. Kirdani and W. R. Slaunwhite, *J. Org. Chem.*, **26**, 2148 (1961); (b) R. Knuppen and H. Breuer, *Ann.*, **639**, 194 (1961).

(9) 7α-Hydroxyestrone: (a) J. Iriarte and H. J. Ringold, *J. Am. Chem. Soc.*, **80**, 6105 (1958); (b) R. Knuppen, O. Haupt, and H. Breuer, *Steroids*, **3**, 123 (1964).

(10) 11α-Hydroxyestrone 3-methyl ether (VI) has recently been prepared in these laboratories (unpublished results from E. Denot, F. Alvarez, E. Necoechea, M. J. Durazo, P. Crabbé, and A. Bowers).

(11) 11β-Hydroxyestrone: R. Knuppen and H. Breuer, *Biochim. Biophys. Acta*, **58**, 147 (1962).

(12) 16-Hydroxyestrones: W. R. Biggerstoff and T. F. Gallagher, *J. Org. Chem.*, **22**, 1220 (1957).

(13) J. Fishman, *J. Am. Chem. Soc.*, **80**, 6105 (1958).

(14) *Inter alia:* (a) J. N. Shoolery and M. T. Rogers, *J. Am. Chem. Soc.*, **80**, 5121 (1958); (b) G. Slomp and B. R. McGarvey, *ibid.*, **81**, 2200 (1959); (c) J. S. G. Cox, E. O. Bishop, and R. E. Richards, *J. Chem. Soc.*, 5118 (1960); (d) R. F. Zürcher, *Helv. Chim. Acta*, **44**, 1380 (1961); **46**, 2054 (1963); (e) J. C. Jacquesy, J. M. Lehn, and J. Levisalles, *Bull. soc. chim. France*, 2444 (1961); (f) Y. Kawazoe, Y. Sato, M. Natsume, H. Hasegawa, T. Okamoto, and K. Tsuda, *Chem. Pharm. Bull.* (Tokyo), **10**, 338 (1962); (g) Y. Kawazoe, Y. Sato, T. Okamoto, and K. Tsuda, *ibid.*, **11**, 328 (1963); (h) T. Okamoto and Y. Kawazoe, *ibid.*, **11**, 643 (1963); (g) K. Tori and E. Kondo, *Tetrahedron Letters*, **No. 10**, 645 (1963).

(15) W. G. Dauben and G. A. Boswell, *J. Am. Chem. Soc.*, **83**, 5003 (1961).

(16) See (a) W. S. Johnson and W. F. Johns, *ibid.*, **79**, 2005 (1957); (b) F. Sondheimer, S. Burstein, and R. Mechoulam, *ibid.*, **82**, 3209 (1960); (c) G. von Mutzenbecher and C. Djerassi, *Proc. Chem. Soc.*, 377 (1963).

(17) A. I. Laskin and J. Fried, U. S. Patent 3,115,444 (Dec. 24, 1963); A. I. Laskin, P. Grabowitch, B. Junta, C. de Lisle Meyers, and J. Fried, *J. Org. Chem.*, **29**, 1333 (1964).

(18) See (a) D. J. Collins, J. J. Hobbs, and S. Sternhell, *Tetrahedron Letters*, **No. 4**, 197 (1963); (b) T. A. Wittstruck, S. K. Malhotra, and H. J. Ringold, *J. Am. Chem. Soc.*, **85**, 1699 (1963).

this long-range coupling to occur the important stereochemical requirements are that the bonds of the coupling protons, *i.e.*, C-4 H and C-6 H, maintain an angle approaching 90°.[19] Since the aromatic C-4 proton in estrone (Ia) or the diacetate (Ic) holds this stereochemical relationship with the 6β-proton, similar long-range coupling in these compounds is to be expected. Normally, the C-4 proton resonance in estrone derivatives is a broad singlet, due to long-range coupling of this proton with protons at C-1 ($J < 1$ c.p.s.), C-2 ($J_{2,4}$ = *ca.* 2 c.p.s.), and C-6β ($J_{4,6}$ = *ca.* 2 c.p.s.), as is observed for Ia and Ic, for example (Experimental). However, the C-4 proton resonance in the diacetate (Vc) appears as a well-resolved doublet ($J_{2,4}$ = 2.5 c.p.s., $J_{1,4}$ small). The resolution of this resonance into a doublet is indicative of the absence of 6β-proton in this substance (Vc), and therefore shows that fermentation has introduced a 6β-hydroxyl group.

Confirmation of this point was obtained chemically. Methylation of 6-ketoestradiol (IVa)[20] afforded the corresponding methyl ether (IVb). As indicated by Wintersteiner and Moore,[21] the preparation of epimeric 6-hydroxyestradiols by sodium borohydride reduction of the 6-keto derivative gives the 6α-hydroxy epimer, whereas catalytic reduction of the 6-keto grouping with platinum oxide in ethanol solution provides the 6β-epimer. Indeed, sodium borohydride reduction of 6-ketoestradiol 3-methyl ether (IVb) gave a diol (Va),[22] which was different from the catalytic hydrogenation product (Vb). Furthermore, the physical properties of the latter (Vb), obtained by catalytic hydrogenation of IVb, were identical with those of the compound isolated from the microbial incubation of estradiol 3-methyl ether (IIb).[23]

It is worthwhile to emphasize that modification of the function at C-3 radically changed the position and the stereochemistry of microbiological hydroxylation of the estrane molecule with *Fusarium moniliforme*.

Experimental[24]

Isolation of the Incubation Product of Estrone (Ia) with *Fusarium monilliforme*.—The surface growth of *Fusarium moniliforme* obtained on Czapek's agar slants after 5 days of incubation at 25° was suspended in 5 ml. of sterile water. Two-milliliter portions of this suspension were used to inoculate 200 ml. of Czapek's broth suspended in a 1-l. erlenmeyer flask. After 18–24 hr. of growth under submerged conditions (rotary shaking at 250 r.p.m.), the culture was used to inoculate 9 l. of the same nutrient solution contained in a 14-l. jar fermentor unit provided with aeration and agitation devices. Along with the inoculum, 1 g. of estrone (Ia) in 25 ml. of dioxane solution was added. Incubation period was extended to 18–20 hr. and the culture was then extracted three times with 10 l. of methylene dichloride. The crude extract was chromatographed on 50 g. of neutral alumina. Elution of the column with benzene–chloroform (95:5) gave first 451 mg. of starting material (Ia). Further elution of the column with benzene–chloroform (80:20) afforded 340 mg. of a crystalline product, m.p. 223–228°. After decoloration with Norit and crystallization from acetone–hexane, the analytical sample of **3,15α-dihydroxyestra-1,3,5(10)-trien-17-one (Ib)** was obtained: m.p. 232–233°; $[\alpha]D$ +212° (*c* 1, EtOH); λ_{max}^{EtOH} 282 mμ (log ε 3.30); ν_{max}^{KBr} 3356–3333 (large), 1736, 1616, and 1592 cm.$^{-1}$.

Anal. Calcd. for $C_{18}H_{22}O_3 \cdot 0.5H_2O$: C, 73.49; H, 7.53; O, 18.96. Found: C, 73.48; H, 7.43; O, 18.97.

Other more polar compounds were also obtained. Unfortunately the very small amounts of these substances did not permit further investigation of their structurss.

3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one Diacetate (Ic).—Acetylation of 200 mg. of the above diol (Ib), in 5 ml. of anhydrous pyridine, with 4 ml. acetic anhydride, for 48 hr. at room temperature gave, after usual work-up, 210 mg. of crystalline material. Further crystallization from acetone–hexane gave 170 mg. of diacetate (Ic): m.p. 144–145°; $[\alpha]D$ +209° (*c* 0.3, $CHCl_3$); λ_{max}^{EtOH} 268 mμ (log ε 2.90), 274–275 mμ (log ε 2.87); ν_{max}^{KBr} 1760, 1735, 1610, and 1254 cm.$^{-1}$ (large); n.m.r., 59.8 (18-H), 125.7 (OAc), 137.0 (OAc), 407 (4-H), 408 and 418 (doublet, 2-H), and 434.5 and 445 c.p.s. (1-H).

Anal. Calcd. for $C_{22}H_{26}O_5$: C, 71.33; H, 7.08; O, 21.59. Found: C, 71.41; H, 7.08; O, 21.42.

3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one 3-Benzoate (Id).—Benzoylation of the diol (Ib) under the conditions described above for the acetylation (benzoyl chloride–pyridine at room temperature) afforded, after usual work-up, the monobenzoate (Id). The analytical sample was obtained by crystallization from acetone–hexane: m.p. 202–203°; $[\alpha]D$ +152° (*c* 0.2, EtOH); λ_{max}^{EtOH} 230–232 mμ (log ε 4.28); ν_{max}^{KBr} 3401, 1735, 1727 (large), 1600, and 1585 cm.$^{-1}$.

Anal. Calcd. for $C_{25}H_{26}O_4$: C, 76.90; H, 6.71. Found: C, 76.72; H, 6.65.

3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one 3-Methyl Ether (Ie).—To a solution of 500 mg. of diol (Ib) in 50 ml. of acetone (twice distilled over anhydrous potassium carbonate), 3.4 g. of anhydrous potassium carbonate and 1.8 ml. of freshly distilled dimethyl sulfate were added. The reaction mixture was gently refluxed for 20 hr. under anhydrous conditions. The potassium carbonate was filtered, and the solvent was evaporated to dryness *in vacuo*. The amorphous residue was chromatographed on 25 g. of neutral alumina. Elution with benzene furnished 250 mg. of crystalline material, m.p. 130–149°. The analytical sample of methyl ether (Ie) was obtained by crystallization from acetone–hexane: m.p. 153–154°; $[\alpha]D$ +205° (*c* 0.9, CH_3OH); λ_{max}^{EtOH} 278 mμ (log ε 3.32) and 287 mμ (log ε 3.29); ν_{max}^{KBr} 3448, 1733, and 1600 cm.$^{-1}$; n.m.r.: 56.0 (18-H) and 226.0 (O-CH_3), *ca.* 175 (6-H, broad multiplet), 398 (4–H), *ca.* 398–407 (2-H), and 427–436 c.p.s. (1-H, doublet).

Anal. Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05; O, 15.98. Found: C, 75.52; H, 8.03; 0.16.19.

3,15α,17β-Trihydroxyestra-1,3,5(10)-triene (IIc).—To a solution of 300 mg. of keto diol (Ib) in 100 ml. of isopropyl alcohol, 1 g. of sodium borohydride in 5 ml. of water was added. The reaction mixture was heated under reflux for 20 hr. The solution was cooled to room temperature, and a dilute hydrochloric acid solution (10%) was added until acid to litmus. Further extraction with ethyl acetate followed by usual work-up gave an amorphous product. This material was chromatographed on 10 g. of neutral alumina. Elution with chloroform afforded 230 mg. of crystalline triol (IIc), m.p. 240–243°. Further crystallization from ethyl acetate gave the pure triol (IIc): m.p. 248–250°; $[\alpha]D$ +148° (*c* 0.5, EtOH); ν_{max}^{KBr} 3.360 (large) and 1618 cm.$^{-1}$. This compound was shown to be identical with the triol (IIc), obtained by direct incubation of estradiol (IIa, *vide infra*). The mixture melting point was not depressed. The infrared curves were superimposable and the polarity by thin layer chromatography was the same.

3,15α,17β-Trihydroxyestra-1,3,5(10)-triene 3-Methyl Ether (IId).—To a solution of 265 mg. of Ie in 21 ml. of methanol was added a solution of 164 mg. of sodium borohydride in 16 ml. of

(19) Applications of this finding have recently appeared: (a) J. C. Orr, M. L. Franco, A. D. Cross, and F. Sondheimer, *Steroids*, **3**, 1 (1964); (b) O. Halpern, P. Crabbé, A. D. Cross, I. Delfin, L. Cervantes, and A. Bowers, *ibid.*, **3**, (June, 1964).

(20) See, for example, B. Longwell and O. Wintersteiner, *J. Biol. Chem.*, **133**, 219 (1940), and references cited therein.

(21) O. Wintersteiner and M. Moore, *J. Am. Chem. Soc.*, **81**, 442 (1959).

(22) H. Breuer and R. Knuppen, *Biochim. Biophys. Acta*, **39**, 408 (1960).

(23) The formation of 6β-hydroxy and 6-keto derivatives of estradiol by mouse liver microsomes has been reported [G. C. Mueller and G. Rumney, *J. Am. Chem. Soc.*, **79**, 1004 (1957)].

(24) Microanalyses were carried out by Dr. A. Bernhardt, Max Planck Institut, Mülheim, Germany, or by Midwest Microlaboratories Inc., Indianapolis, Ind., U. S. A. Rotations were taken at room temperature, for the sodium D-line. Melting points were determined in capillary tube with a Mel-Temp apparatus. They are corrected. Infrared spectra were taken with a Perkin-Elmer Model 21, equipped with a NaCl prism. Ultraviolet absorption spectra are for 95% ethanol solutions and were measured with a Beckman spectrophotometer, Model D. U. N.m.r. spectra were recorded at 60 Mc.p.s. using 5–8% w./v. solutions of steroid in chloroform containing tetramethylsilane (TMS) as an internal reference. Resonance frequencies, ν, are quoted as c.p.s. downfield from the TMS reference (0.0 c.p.s.) and are accurate to ±1 c.p.s. Coupling constants, *J*, also expressed in c.p.s. units, are accurate to ±0.5 c.p.s. We are grateful to the Universidad Nacional Autonoma de México for time on the A-60 spectrometer.

methanol. The reaction mixture was allowed to stand 15 hr. at room temperature after which time a dilute acetic acid solution (10%) was slowly added until the solution was acidic. Water was then added and the organic compound was extracted with ether. The organic layer was washed with water until neutral, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness *in vacuo*. The residual material was purified by crystallization from acetone–hexane affording 210 mg. of a pure sample of diol (IId): m.p. 141–142°; $[\alpha]_D$ +154° (*c* 0.16, $CHCl_3$); λ_{max}^{EtOH} 278–280 mμ (log ε 3.31), 287 mμ (log ε 3.27); ν_{max}^{KBr} 3472–3268 and 1610 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67; O, 15.87. Found: C, 74.93; H, 8.56; O, 15.56.

Incubation of Estradiol (IIa) with *Fusarium moniliforme*.—The mixture obtained after fermentation of 2 g. of estradiol (IIa) with *Fusarium moniliforme* under the above reported conditions gave by chromatographic separation on 100 g. of neutral alumina 440 mg. of crude triol (IIc). The analytical sample of this triol (IIc) was obtained by crystallization from acetone–hexane: m.p. 250–251°; $[\alpha]_D$ +150° (*c* 0.3, EtOH); λ_{max}^{EtOH} 280–282 mμ (log ε 3.32); ν_{max}^{KBr} 3390–3279 (large), 1618, and 1585 cm.$^{-1}$.

Anal. Calcd. for $C_{18}H_{24}O_3$: C, 74.97; H, 8.39; O, 16.64. Found: C, 75.10; H, 8.69; O, 16.65.

This substance was shown to be identical with an authentic sample of triol (IIc)[17] by mixture melting point, infrared spectra, and thin layer chromatography.

Other substances were also obtained during this incubation experiment. Unfortunately, the minute amount which was isolated did not allow further investigation of their structure.

3-Hydroxy-14-isoestra-1,3,5(10),15-tetraen-17-one (III).—A solution of 100 mg. of the ether (Ie) in 3 ml. of dry pyridine was cooled in an ice bath and treated, dropwise, with 0.7 ml. of phosphorus oxychloride. The mixture was allowed to stand at room temperature for 24 hr. and the resulting yellow mixture was decomposed, at 0°, by cautious addition of water and dilute hydrochloric acid. This solution was extracted with ethyl acetate several times. The organic layer was washed first with 10% hydrochloric acid solution, then with water until neutral, and dried over anhydrous sodium sulfate. After filtration and evaporation, there was obtained an oily product which was chromatographed on 10 g. of neutral alumina. Elution with hexane–benzene (90:10) afforded a low-melting crystalline material showing ultraviolet absorption at *ca.* λ_{max} 221–223 mμ, indicating a probable mixture of 15-dehydroestrone 3-methyl ether and 15-dehydro-14-isoestrone 3-methyl ether (III).[16a] Further crystallization from acetone–hexane afforded a compound showing the physical properties of the 15-dehydroisoestrone derivative[16a] (III): m.p. 95–96°; $[\alpha]_D$ +408° (*c* 0.25, $CHCl_3$); λ_{max}^{EtOH} 221 mμ (log ε 4.18), 278 mμ (log ε 3.37), 288 mμ (log ε 3.35); ν_{max}^{KBr} 1703 and 1616 cm.$^{-1}$.

Incubation Product from Estradiol 3-Methyl Ether (IIb).—The mixture obtained from incubation of 2 g. of estradiol 3-methyl ether (IIb), under the conditions reported above for the fermentation of estrone, was filtered over alumina to separate the unreacted material from the hydroxylated compound. There was obtained 530 mg. of crude diol ether (Vb) which was purified by chromatography over 30 g. of Florisil. Elution of the column with benzene–chloroform (80:20) gave a crystalline product, m.p. 185–191°, as main constituent. Further purification by crystallization from acetone–hexane gave 325 mg. of pure diol ether (Vb): m.p. 190–192°; $[\alpha]_D$ +45° (*c* 0.5, MeOH); λ_{max}^{EtOH} 278 mμ (log ε 3.76); ν_{max}^{KBr} 3280 (large) and 1613 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67; O, 15.87. Found: C, 75.36; H, 8.64; O, 16.27.

3,6β,17β-Trihydroxyestra-1,3,5(10)-triene 3-Methyl Ether 6β,17β-Diacetate (Vc).—To a solution of 135 mg. of diol (Vb) in 5 ml. of pyridine, 5 ml. of acetic anhydride was added. The reaction mixture was kept overnight at room temperature. Water was then added, and the organic compound was extracted with ethyl acetate. The organic layer was washed with 10% hydrochloric acid, until acidic, then with 5% sodium carbonate solution and finally with water. After drying over anhydrous sodium sulfate, the organic solution was filtered and evaporated to dryness. The crude product (240 mg.) was chromatographed on 10 g. of neutral alumina. Elution with hexane–benzene (1 l.) gave the crystalline diacetate (Vc) which was recrystallized from methanol–water: m.p. 108.5–110.5°; $[\alpha]_D$ +85° (*c* 0.6, $CHCl_3$); λ_{max}^{EtOH} 268 mμ (log ε 3.92); ν_{max}^{KBr} 1736, 1613, and 1250 cm.$^{-1}$; n.m.r., 52.1 (18-H), 124.1 (OAc), 227.3 (OCH_3), 411.0 (4-H, doublet $J_{2,4}$ = 2.5 c.p.s.), *ca.* 411–418 c.p.s. (2-H).

Anal. Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82; O, 20.70. Found C, 71.62; H, 7.74; O, 20.98.

3,17β-Dihydroxyestra-1,3,5(10)-trien-6-one 3-Methyl Ether (IVb).—To a solution of 200 mg. of phenol (IVa) in 20 ml. of acetone (twice distilled over anhydrous potassium carbonate), 1.3 g. of anhydrous potassium carbonate, and 0.7 ml. of freshly distilled dimethyl sulfate was added. The reaction mixture was gently refluxed for 20 hr. under anhydrous conditions. The reaction flask was cooled, the potassium carbonate was filtered, and the solvent was evaporated under reduced pressure. The crude product was chromatographed on 10 g. of neutral alumina. Elution with benzene gave 190 mg. of crystalline methyl ether (IVb). By recrystallization from acetone–hexane, the analytical sample of ether (IVb) was obtained: m.p. 129–130°; $[\alpha]_D$ −23° (*c* 0.28, $CHCl_3$); λ_{max}^{EtOH} 222 mμ (log ε 4.34), 254–256 mμ (log ε 3.94), 324 mμ (log ε 3.48); ν_{max}^{KBr} 3400, 1681, and 1608 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05; O, 15.98. Found: C, 75.92; H, 8.04; O, 15.99.

3,6α,17β-Trihydroxyestra-1,3,5(10)-triene 3-Methyl Ether (Va).—The reduction of 50 mg. of the 6-ketone (IVb) in 40 ml. of methanol with 32 mg. of sodium borohydride, 15 hr. at room temperature, followed by usual work-up, afforded a crystalline diol. This substance (Va) was purified by crystallization from acetone–hexane to give m.p. 102–104°; $[\alpha]_D$ +88° (*c* 0.2, $CHCl_3$); λ_{max}^{EtOH} 279 mμ (log ε 3.36), 287 mμ (log ε 3.28); ν_{max}^{KBr} 3448–3226 and 1610 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{26}O_3 \cdot H_2O$: C, 71.22; H, 8.81. Found: C, 71.28; H, 9.08.

3,6β,17β-Trihydroxyestra-1,3,5(10)-triene 3-Methyl Ether (Vb).—A solution of 100 mg. of keto steroid (IVb) in 100 ml. of absolute ethanol was hydrogenated in presence of 74 mg. of prereduced platinum oxide. After take-up corresponding to 1 equiv. of hydrogen, the catalyst was filtered and the solvent was evaporated under reduced pressure. The crude reduction product was chromatographed on 3 g. of neutral alumina. Elution of the column with benzene–chloroform (3:1) gave a crystalline material, m.p. 175–180°. Further crystallizations from acetone–hexane gave a small sample of pure 6β-hydroxyestradiol 3-methyl ether (Vb), m.p. 192–193°. This substance was homogeneous on a chromatoplate, and its polarity was the same as that of the compound (Vb) obtained by microbial incubation of estradiol 3-methyl ether (*vide supra*). The mixture melting point was not depressed and the infrared curves were superimposable.

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