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Microbial Synthesis of Sex Hormones*

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Abstract—Estrone, which is obtainable from 19-hydroxycholesterol acetate through microbial oxidation, was chemically transformed to orally active estrogens, ethynylestradiol and methylestradiol. For progestin synthesis, 5α-bromo-6, 19-oxidoisoandrosterone was harvested from the culture broth(ATCC:19170), in which 5α-bromo-6, 19-oxidocholestanol acetate was added as a carbon source. Methylestrenolone, a potent orally active progestin, was synthesized from the harvested product via several reactions. Norethindrone, a popular progestin, was also prepared from 19-norandrostenedione. 19-Norandrostenedione is also available through microbial oxidation and chemical modification of 5α-chlor-6, 19-oxidocholestanol acetate.

It has been known since Sohngen's experiment in 1913 that some microbe can utilize cholesterol as a sole carbon source.[1] However systematic investigations have been carried out recently. Now overall metabolic pathway of androst-4-ene-3, 17-dione (**I**) to carbon dioxide and water is visualized(scheme 1)[2-7].

On the other hand, a degradation mechanism of cholesterol side chain was studied by conventional[8] and also by radioactive labelling experiments[9]. It is now clear that the side chain of cholesterol(**II**) is first oxidized to C_{26} or C_{27} carboxylic acid via hydroxylation, and then successive β-oxidation was followed(scheme 2).

However, it is believed that there is no compulsory order between stroidal ring fission and its side chain degradation. An evidence for this belief is from the following facts.

1) When cholesterol was incubated with a soil microbe, there is neither accumulation of side chain cleaved products nor ring fission products.
2) Many sterols(**III**) which have different side chains from cholesterol were degradated to indanepropionic acid derivatives(**IV**) and these indanepropionic acids had their original intact side chains.[8]
3) When ring fission was inhibited by a structural modification, such as hydroxylation

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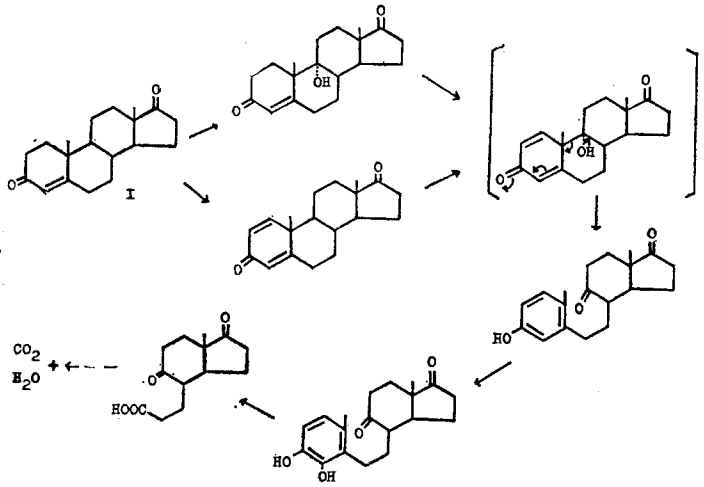
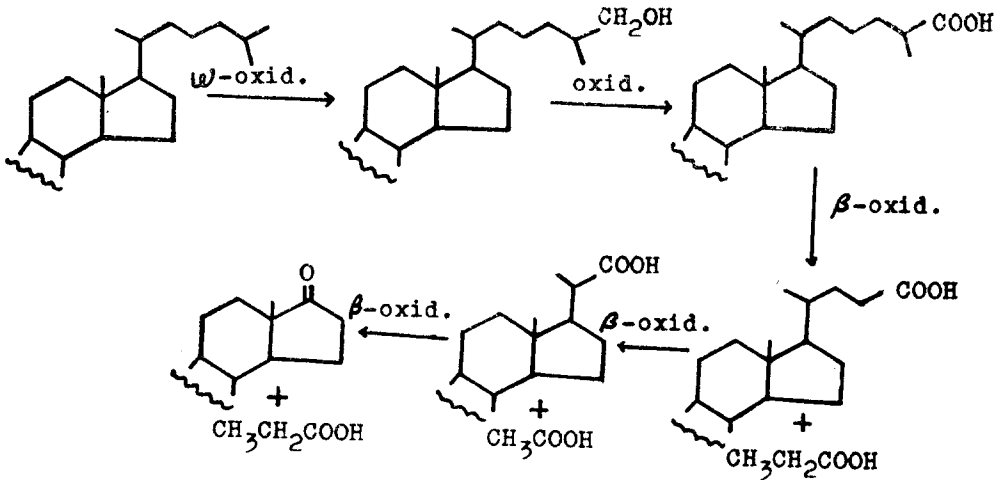
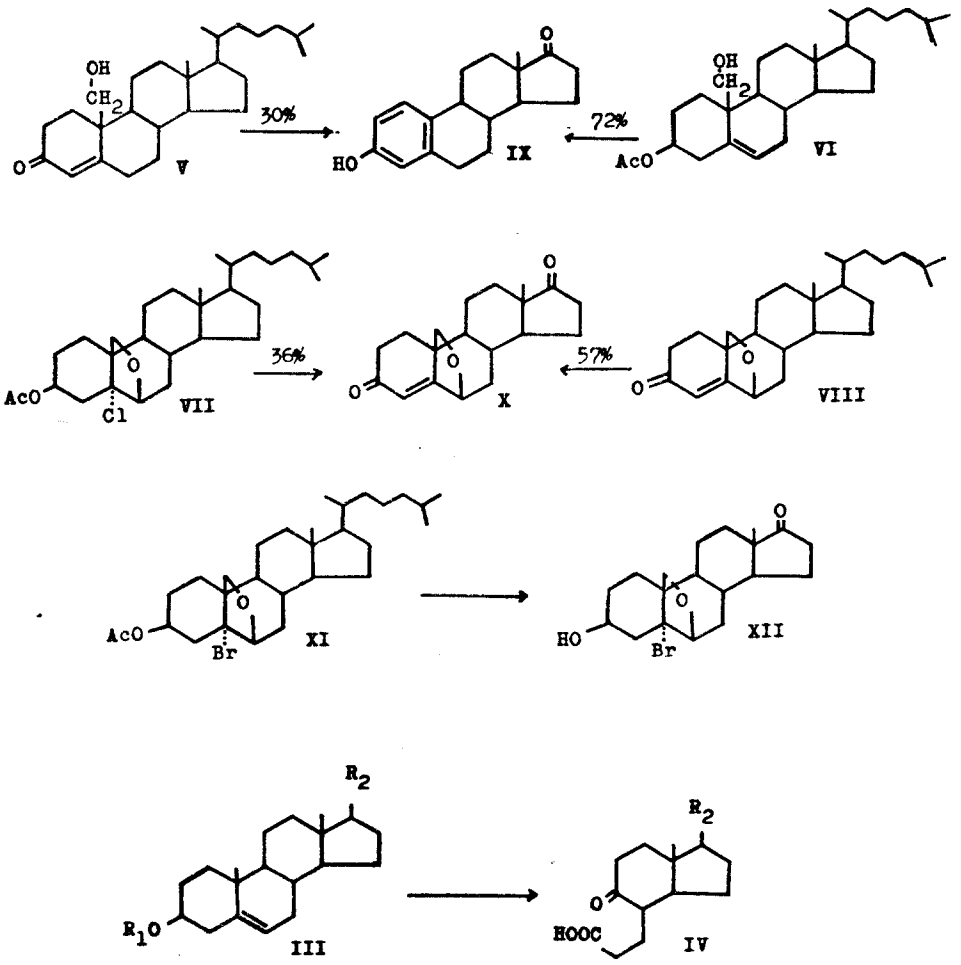
of 19-methyl(**V, VI**) or introduction of oxido-ring between C_6-C_{19}(**VII, VIII**), the side chains of cholesterol derivatives were completely oxidized to 17-ketosteroids(**X**) (scheme 3).



Scheme 1



Scheme 2



Scheme 3

R_1: H or CH_3CO
R_2: Various carbon chain excluding cholesterol side chain(C_8H_{17})

If one carefully examines the Scheme 3, it can easily be noticed a peculiar phenomenon. When estrone(**IX**) was produced by a microorganism, CSD-10(ATCC: 19170), the existance of α, β unsaturated keto-system in substrate structure was less favorable than that of 3β-acetoxy function in it.

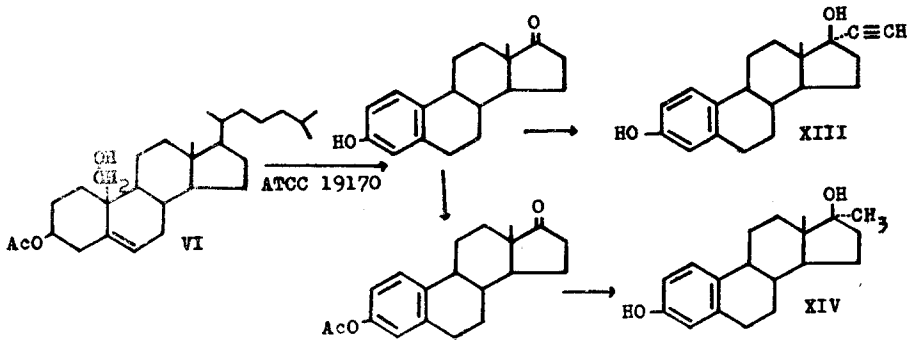
On the other hand, when 6, 19-oxidoandrost-4-en-3, 17-dione was produced by the same organism, 3β-acetoxy function was much less favorable than α,β-unsaturated keto-system. This discrepancy was clearly illustrated by 5α-halogen effect on 3β-hydroxy group oxidation.[11]

Furthermore, substitution of 5 α-chlorine to 5 α-bromine(**XI**) in 6, 19-oxidocholestane skeleton made the accumulation of main meta bolic product, 5 α-bromo-6, 19-oxidoisoandrosterone(**XII**), instead of compound **X**. The compound **XII** would be an attractive intermediate for orally active 19-norsteroid synthesis.

It is accordingly an object of the present study to provide new and improved methods for preparing orally active estrogens and progestins from cholesterol derivatives. The concept of the processes resides in an application of microbial oxidation, transformation and chemical modification of steroids.

Synthesis of Orally Active Estrogens—19-Hydroxycholesterol acetate(**VI**), a substrate for microbial conversion to estrone(**IX**), was easily obtained via three step chemical reactions.[12] A large scale fermentation was carried out for estrone synthesis. Incubation, extraction and purification procedure were followed by a method which was mentioned previously.[8] For the chemical synthesis of orally active ethinylestradiol(**XIII**)[13], Inhoffen's method [14,15] was successfully applied. From 3 g of estrone, 3 g of 17 α-ethinyl-estradiol, which showed a single spot on a thin layer chromatoplate, was obtained. For the chemical synthesis of 17α-methylestradiol(**XIV**),[16] the acetylation of estrone was attempted before methylation, because the solubility of estrone in ether and toluene is poor.

After acetylation with pyridine-acetic anhydride mixture solution, methylation was successfully carried out in 80% yield by adding excess amount of methyl magnesium bromide(scheme 4).

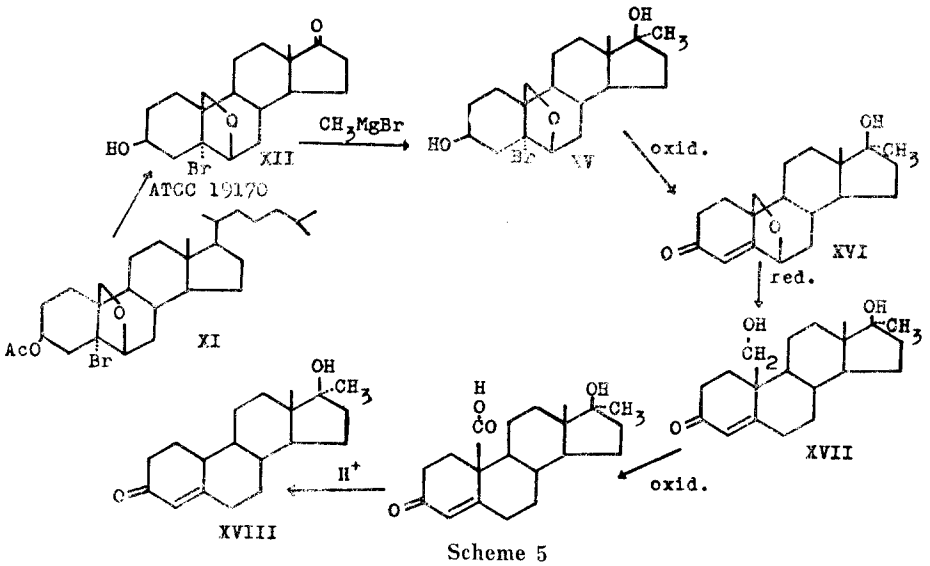


Scheme 4

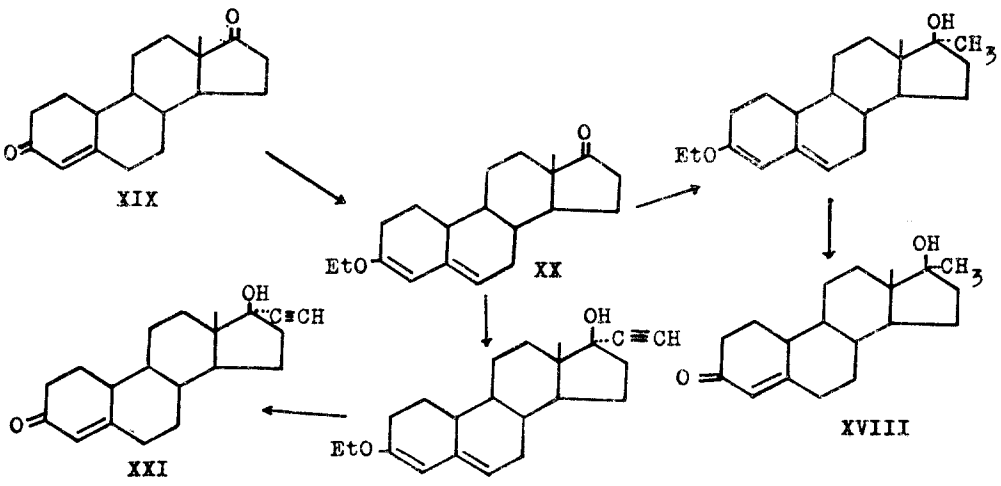
Synthesis of Orally Active Progestins a) Methylestrenolone(17 α-methyl-17 β-hydroxyestr-4-ene-3-one)(XVIII)—As a substrate for microbial conversion to a 17-ketosteroid which will be a suitable intermediate for the synthesis of orally active progestin, 5 α-brome-6, 19-oxidocholestanol acetate(XI) was prepared.[12] The compound XI was add edinto the culture broth ofCSD-10(ATCC: 19170) in 300r/ml concentration. After 90 hour incubation on a rotary shaker, the broth was extracted and application of silica gel chromatography gave 5 α-bromo-6, 19-oxidoisoandrosterone(**XII**) in 55% yield. The compound **XII** was directly methylated and then oxidized to 6, 19-oxido-17 α-methylandrost-4-ene-3-one-17 β-ol

(**XVI**). This compound was further reduced to 17 α-methylandrost-4-ene-17 β-19-diol-3-one(**XVII**) and oxidized again to methylestrenolone(**XVIII**)(scheme 5). On the other hand, 19-norandrost-4-ene-3, 17-dione(**XIX**), which is obtainable through microbial conversion and chemical modification from cholesterol, was also used as an intermediate for methyl estrone synthesis. For the derivatization of C_{17}-position of compound **XIX**, 3-oxo-function was protected by an enol ether formation. The enol ether(**XX**) was prepared in good yield with ethylorthoformate in the presence of p-toluene-sulfonic acid.

Methylation of 17-keto group with methyl magnesium bromide provided a methylation



Scheme 5



Scheme 6

product, methyl estrenolone. (scheme 6)

b) Norethindrone(17 β-hydroxy-19-nor-17α-pregn-4-en-20-yn3-one (XXI)—Enol ether (**XX**) was converted to Norethindrone by Djerassi's[17] method in which ethinyl group was introduced in the presence of potassium t-amylalcohol, (scheme 6)

DISCUSSION

Ethynylestradiol was nearly quantitatively prepared from estrone, though the experimental procedure was not simple.

For the preparation of 17α-methylestradiol, the direct methylation of estrone was not successful because of its insolubility in toluene and ethyl ether. In order to increase its solubility, estrone was acetylated before methylation. In this case, methylation was so smooth that Girard's reagent T treatment[16] for the elimination of non-methylation material was omitted.

A new route for the synthesis of methylestrenolone, which is an active progestin by oral administration, from 5α-bromo-6, 19-oxidoisoandrosterone(**XII**) was developed. The compound **XII** is a microbial conversion product from 5α-bromo-6, 19-oxidocholestanol acetate(**XI**) in this work. In a typical experiment, the yields of compound **XII** from compound **XI** were about 55%. However, these yields are believed to be incresed by improving the fermentation conditions.

Though methylestrenolone was easily obtained from compound **XII** after five steps of chemical reactions, norethyndrone was not btained by same line of reaction steps. Toerefore, another intermediate for norethyhdrone synthesis was chosen. Thus 19-norandrost-4-ene-3, 17-dione(**XIX**) which is obtainable from 6, 19-oxidoandrost-4-ene-3, 17-dione(**XIX**)[12] was converted to enole ther(**XX**) and successfully ethynylated.

EXPERIMENTAL

Melting points are not corrected. Infrared spectra were recorded on a Beckman Spectrophotometer Model 4. Thin Layer Chromatography was followed by Stahl[18]

Materials—Culture strain: CSD-10(ATCC: 19170) was maintained and grown in a salts medium or in a nutrient broth consisting of as follows:

salts medium……KH_2 PO_4, 0.1%; K_2HPO_4, 0.1%; NH_4 NO_3, 0.1%; $MgSO_4$, 0.2%; $CaCl_2$, 0.002%; $FeCl_3$, 0.005% and cholesterol 0.03%(1% concentration in dimethylformamide).

nutrient broth……Difco Nutrient Broth(dehydrated) 8%.

All of thesolvents and organic and inorganic chemicals were reagent grade. Petroleum ether refers to the fraction with a boiling point of 40 to 70°. Silica gel(E. Merck: 7729) was used for column chromatography, and Silica gel HF(E, Merck 7741) was used for thin-layer chromatography. Cholesterol was extracted from cattle brains(18% yield in dry

weight). Dehydroisoandrosterone, estrone, 19-norandrost-4-ene-3, 17-dionewere obtained from Searle Chemicals, Inc, (Chicago, U.S.A.). Methyl magnesium bromide(3 mol in 1 liter of n-buthyl ether) was supplied by Junsei Pure Chemicals & Co. (Tokyo, Japan). Metachloroperbenzoic acid was supplied by FMC coporation, Inorganic chemicals Div. (New Corteret, N. J., U.S.A.)

Microbial Synthesis of Estrone(IX)—19-Hydroxycholesterol acetate(**VI**) was prepared from cholesterol acetate after three step chemical reactions. Compound **VI** was converted to estrone by CSD-10.[8]

Synthesis of Ethynylestradiol(XIII)—After one liter three necked flask was dipped in an acetone-dry ice bath, about 250 ml of liquid ammonia was condensed in the flask. 7.5 g Of metal potassium was cut to a few pieces and put into the flask. The colorless liquid ammonia was immediately turned blue. Acetylene gas was passed until the blue color had disappeared. Then 3 g of estrone suspended in 150 ml of thiophen free benzene and 50 ml of dehydrated ethyl ether was added slowly through a dropping funnel under stirring. The freezing mixture was then removed, and the whole reaction mixture was allowed to stand for about 2 hours, and was further stirred overnight for evaporation of liquid ammonia. Thereupon the reaction mixture was treated with ice and water, acidified with sulfuric acid to an acid reaction to Congo red and the solution was extracted with excess amount of ethyl ether. The etheral solution was washed with water, 5% sodium bicarbonate and again with water until washing water was neutral.

Then the ether was evaporated. The residue was dissolved in a little amount of methanol and diluted with water.

A fine needle form of crystals was come out. The yields amounted to 2.7g(m.p. 142-144°). The mother liquid was warmed up and a small amount of water was again added.

The second crop amounted to 0.4 g and further recrystallization gave 0.3 g of ethnnyl-estradiol.

Both of first and second crops showed a single spot on a thin-layer plate(solvent;chloroform: acetone, 4:1, coloring agent: 50% H_2SO_4).

Synthesis of 17 α-Methylestradiol(XIV)—3 g Of estrone was dissolved in a mixture of 6 ml of acetic anhydride and 6 ml of pyridine by warming up around 40° for a while, and then stood at room temperature for 12 hours. Distillation of the solvent mixture under reduced pressure and then air ventilation gave estrone acetate quantitatively which showed a single spot on a thin-layer plate. Without further purification, the reaction product was used for mythylation.

A solution of 1.15 g of estrone acetate in 40 ml of dry toluene was added slowly with stirring to a mixture of 10ml of methyl magnesium bromide(3 mol in 1 liter n-buthyl ether) and 10 ml of ethyl ether under nitrogen atmosphere. After gently refluxing for 3 hours, the reaction mixture was stood overnight. The Grignard complex was then destroyed with 2% NH_4Cl solution containing hydrocloric acid, and the organic material was taken up in

ethyl ether. The etheral solution was washed once with 10% $NaHCO_3$, and then with water, dried and evaporated, whereupon the residue was turned out to crystalline form. Recrystallization from aquaous methanol gave 0.94 g of methylestradiol(m.p. 190-193°) which showed a single spot on a silica gel thin-layer plate.

Microbial Synthesis of 5 α-Bromo-6, 19-oxidoisoandrosterone(XII)—CSD-10 stock culture was transfered into a mineral salts medium enriched with cholesterol in 300 ml Erlenmeyer flask. After incubation on a rotary shaker(1 inch stroke, 120 rpm, r.t.) for 4 days, 30 ml of the culture medium was divided equally into 6 of the salts medium in 300 ml Erlenmeyer flask again, and incubation was kept going. A large scale fermentation was initiated by the addition of 40 m*l* of the 4-day old CSD- 10 culture medium into each of 6 two liter Erlenmeyer flasks in which 400m*l* of nutrient broth was held. At zero time of the fermentation, 720 mg of 5 α-bromo-6, 19-oxidocholestanol acetate(**XI**) dissolved in 30 m*l* of dimethylformamide was also divided into the 6 flasks.

After 90 hour fermentation under the same condition as mentioned above, the broth was acidified with acetic acid. The precipitated cell debris was filtered through Celite layer on a Buchner funnel, and the filter cake was washed with a small amount of chloroform. The filterate was extracted repeatedly with chloroform. The whole chloroform extract was washed with water, dried over sodium sulfate and evapolation of the solvent gave non crystallizable residue. The residue was dissolved again in a small amount of chloroform and applied over asilica gel column(2.5×35 cm, silica gel 60 g, Celite 3g). The column was eluxed with chloroform until a hydrolysis product, 5 α-bromo-6, 19-oxidocholestanol was cluted out. Then 0.3% methanol in chloroform was chosen as an eluent. The fraction in which the product, 5 α-bromo-6, 19-oxidoisoandrosterone was contained, was collected. Evapolation of the solvent under reduced pressure gave 240 mg of the product which showed m.p. around 200°. The ir spectrum of this compound was identifical to an anthenic sample, prepared from dehydroisoandrosterone[12], and a mixed melting point determination showed no depression.

5 α-Bromo-6, 19-oxido-17 α-methylandrostane-3, 17 β-diol(XV)—As compound **XII** was poorly soluble in ethyl ether, it was acetylated before methylation as did for methylation of estrone. 1g Of the acetylation product, 3 β-acetoxy-5 α-bromo-6, 19-oxidoandrostane-17-one, was dissolved in 40 m*l* of toluene, and added into a mixture of 20 m*l* of methyl magnesium bromide reagent and 10 m*l* of ethyl ether through a dropping funnel. After 3 hours of gentle reflux, the reaction mixture was kept overnight at room temperature. The Grignard complex was carefully destroyed with hydrochloric acid and extracted with a chloroform-ethylene chloride mixture. The organic solvent layer was washed with water, dried and evaporation of the solvent gave crystalline residue. Though this residue contained a small amount of non-reacted material, recrystallization from acetone, pet. ether gave 800 mg of the compound **XV**(m.p. 190-191°). Ir spectrum λ_{max}: 2.86, 2.89, 6.14, 6.71, 6.93, 7.30, 7.49μ.

17 α-methyl-6, 19-oxidoandrost-4-en-17 β-ol-3-one(XVI)—600 mg Of the compound **XV**, dissolved in 15 m*l* of dioxane and 30 m*l* of acetone, was kept near 0°, and 0.9 m*l* of 8 N-chromoxide(**VI**) in sulfuric acid(23 m*l* of c-H_2SO_4 in 100 m*l*) was added dropwise under stirring. 25 Minute after, an excess chromoxide(**VI**) was quenched with a small amount of ethanol and the reaction micture was diluted with a large volume of water. The extraction was repeatedly carried out with methylene chloride. 200 m*l* Of the combined extract was washed with 5% $NaHCO_3$, water, dried and worked out as usual manner. Recrystallization from acetone-pet. ether gave 460 mg of the compound **XVI**(m.p. 151-152°). I.R. spectrum λ_{max}: 2.81, 2.88, 5.79, 6.24, 6.82, 7.28 μ.

17 α-Methyl-19-hydroxymethylandrost-4-en-17 β-ol-3-one(XVII)—400 mg of compound **XVI** was dissolved in 20 m*l* of 95% acetic acid, and 10 g of activated zinc powder was added. The reaction mixture was kept stirring at 90°-100° for 10 minutes. After cooling, the reaction mixture was filtered and the filterate was reduced its volume under reduced pressure. The condensed solution was dilute with a large volume of water and extracted with ethylene chloride. The organic layer was washed with water, and dried over sodium sulfate. Evaporation of the solvent gave about 400 mg of crystalline residue. Without further purification, the residue was used for the synthesis of methylestrenolone (**XVIII**).

Methylestrenolone(17 α-methylestr-4-en-17 β-ol-3-one(XVIII)—a) The whole crude product, compound XVII was dissolved in a small amount of acetone and was kept near 0°. 0.4 m*l* Of 8 N-chromoxide(**VI**) in sulfuric acid was added dropwise and stood for 25 minutes.

The excess amount of chromoxide(**VI**) was quenched with a small volume of ethanol and then a large excess volume of water was added for dilution. The precipitate was extracted with methylene chloride and the organic layer was worked out as usual manner. Non-crystallizable residue was dissolved in 15m*l* of t-butanol. After adding 1.2 m*l* of concentrated hydrochloric acid, the solution was refluxed for 20 minutes. The reaction mixture was reduced its volume to 7-8 m*l* and then it was diluted with 30 m*l* of benzene, washed with 5% $NaHCO_3$, water and dried over sodium sulfate. Evapolation of the solvent mixture under reduced pressure gave non-crystallizable residue. The residue was disslved in a small amount of chloroform and applied over a small silica gel column. Elution was carried out with 0.2% methanol in chloroform and methylestrenolone fraction was collected. Evapolation of the solvent gave 80 mg of methylestrenolone(m.p. 153-155°).

This compound was identical with an authentic sample which was prepared from 19-norandrostenedione.[17]

b) 1.9g of 3-ethoxy-19-norandrosta-3, 5-diene-17-one(**XX**) was dissolved in 20 ml of ether, and 21 ml of 3 mol methyl magnesium bromide reagent was added carefully under stirring and refluxed gently for two hours. After standing overnight at room temperature, the reaction mixture was poured into ice slurry. After acidification with hydrochloric acid,

the reaction mixture was extracted with an excess amount of ether. Evaporation of the solvent after working out as usual way gave crude crystal (m.p. 140-150°).

Recrystallization from ether, pet. ether gave 1.4g of methylestrenolone(m.p. 154-156°).

Norethindrone(19-nor-17 α-ethynylandrost-4-ene-3-one-17 β-01 (XXI)—C. Jerassi's method was successfully applied. 1 g of enol ether(XX), dissolved in 25 ml of dry toluene was added to a solution of 1g of potassium in 25ml of t-amyl alcohol.

After replacement of the air with nitrogen gas, acetylene gas was then passed through the mixture for 14 hours at room temperature. After dilution with water and adjustment of pH to 1 by adding of hydrochloric acid, steam was passed through the solution until until no more volatile material came over. The solid product which was came out after cooling was collected, and recrystallization from ethyl acetate gave 65% yield of norethindrone (m.p. 202-204°).

3-Ethoxy-19-norandrost-3, 5-dien-17-one(XX)—In 20 g of 19-norandrost-4-ene-3, 17-dione(XIX), dissolved in 100 ml of peroxide-free dioxane and 20 ml of freshly distilled ethyl orthoformate, 2.7 ml of a solution of 4.88 g of p-toluene sulfonic acid monohydrate in 5.4 ml of dioxane and 11 ml of absolute ethanol were added. The solution was shaken occasionally at room temperature for one hour. The solution was then treated with 11 ml of pyridine and the solvents were removed at reduced pressure. The crystalline residue was extracted with ether, washed well with water, dried, evaporated and crystallized from pet. ether-acetone to finish 16.5 g of the enol ether(**XX**) with m.p. 135-141°.

This material was used in the subsequent transformations.

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