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Chromogenic Reactions of Steroids with Strong Acids. VI.[1] Behavior of Phenolic Steroids in Concentrated Sulfuric Acids[2]

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Behavior of various 3-methoxylated phenolic steroids (I—VII and IX—XI) in conc. H_2SO_4 was studied, in particular relation to the earlier period of the Kober reaction. Alteration of the light-absorptions at the maxima of 432, 465, and 515 nm was detected to be varied with the concentrations of H_2SO_4 (Fig. 1). The maximum absorption at 372 nm was observed in the mixture of estradiol-3-methyl ether (II) with 97.2% H_2SO_4 (Fig. 2), which appeared, on the contrary, immediately after mixing III, IV, V, VI, or VII with the same acid (Fig. 3 and 4). Nuclear magnetic resonance study on the solutions of III, IV, or V in the acid (Fig. 5) suggested that the absorption at 372 nm is due to the carbocation χ-372 (VIII) produced. As for the Kober reaction of steroidal estrogens, it was postulated that χ-372 initially formed is oxidized into the characteristic chrommophores χ-465 and χ-515, through which the highly dehydrogenated steroidal olefins[1] are finally produced.

Various steroids are known to produce chromophoric and/or fluorophoric species when they come into contact with various strong acids. Numerous reports on these reactions have thus been published for the qualitative as well quantitative analysis of steroidal compounds. The Kober reaction[4] is the well known chromogenic reaction highly specific to the steroidal estrogens and has long been employed for the determination of these steroids in the field of clinical chemistry. The behavior of rather simple organic molecules in strong acids has recently been clarified by Olah, *et al.* and Deno, *et al.*[5] While the color reactions of steroids with strong acids are numerous in the variety, little work has been reported on understanding their detailed chemistry.[6] Some explanations have ever been proposed for the Kober reaction, which were speculative and deficient in the experimental bases.[7] In the preceding paper of this series, the specificity of this reaction was described and the structural requirements were made clear for the positive Kober reaction of the steroidal molecules.[8] The dehydrogenated and disproportionation products were also obtained from the reaction mixtures of estrone-(3-methoxyestra-1,3,5(10)-trien-17-one) (I) as well as of estradiol-3-methyl ethers (3-methoxyestra-1,3,5(10)-trien-17β-ol) (II) and their chemical structures were elucidated.[1]

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[1] Part V: M. Kimura, M. Kawata, K. Akiyama, K. Harita, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **21**, 1741 (1973).

[2] Preliminary accounts of a part of this work have been published: M. Kimura, K. Akiyama, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **20**, 2511 (1972).

[3] Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*

[4] S. Kober, *Biochem. Z.*, **239**, 209 (1931); *idem, Biochem. J.*, 32, 357 (1938).

[5] For reviews see G.A. Olah and P.v.R. Schleyer, Ed., "Carbonium Ions," Vol. I, Wiley-Interscience, New York, 1968 and Vol. II, 1970.

[6] T. Watanabe, *Eisei Shikenjo Hokoku*, **74**, 111 (1956); *idem, Chem. Abstr.*, **51**, 15339*f* (1957); *idem, ibid.*, **77**, 81, 87 (1959); *idem, Chem. Abstr.*, **55**, 10550*d*, 10503*c* (1961); K. Kato, *ibid.*, **12**, 578, 582, 824, 1319 (1964); T. Momose, Y. Ueda, M. Kageura, T. Masumura, and K. Ohta, *Chem. Pharm. Bull.* (Tokyo), **12**, 1415 (1964).

[7] *a*) W. Zimmermann, "Chemische Bestimmungsmethoden von Steroidhormonen in Körperflüssigkeiten," Springer-Verlag, Berlin, 1955, p. 52; *b*) H.A. Jones and R. Hahnel, *Nature*, **215**, 1381 (1967); *c*) *idem, Steroids*, **13**, 693 (1969).

[8] M. Kimura, M. Kawata, K. Akiyama, K. Harita, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), 21, 1720 (1973).

The present paper deals with the behavior of some phenolic steroids in concentrated sulfuric acid, in particular relation to the earlier period of the Kober reaction.

Result and Discussion

Formation of the carbocation[9] through protonation and/or dehydration can be assumed in the earlier period when steroidal olefin or hydroxysteroid comes into reaction with strong acid. The carbocations in the acid system are considered to show a general tendency that they give rise to the secondary change with decrease in the acidity of the medium,[10] though they are stable under higher acidity. The Kober test is a two step reaction. The first is heating with 50—80% (v/v) sulfuric acid at 100° for about 20 min, and the second step is dilution with water and reheating. The Kober chromophore may, therefore, be derived from such carbocation through some unknown processes. The chromogenic reactions of II which were carried out with sulfuric acid in different concentrations at 100° for 20 min gave absorption spectra as shown in Fig. 1. At the region of higher acidity, the light absorptions at the maxima were in the order of decreasing intensity at $432 > 465 \gg 515$ nm and a tendency of blue shift was observed on the maxima at 465 and 515 nm, which correspond to those shown at the first and second steps of the Kober reaction, respectively. With decreasing in acidity of medium, these absorptions were in the order of decreasing intensity at $432 < 465 > 515$ nm and finally the maximum at 432 nm faded out with increase in the intensity at 515 nm.

In order to investigate the earlier parts of these chromogenic processes, the progress of the reaction was followed by observing the absorption spectra of various steroids in concentrated sulfuric acid at 25°, in which the carbocations probably produced at the earlier stage are considered generally to be stable. When II was dissolved in 97.2% sulfuric acid, the absorption maximum appeared immediately at 384 nm which was shifted to 372 nm in a subsequent short period and then transfered gradually to 456 nm exibiting an isosbestic point at 406 nm. Another absorption maximum at 432 nm also appeared subsequently during the elapse of time as shown in Fig. 2. The wave-length of maximum thus appeared at 456 nm

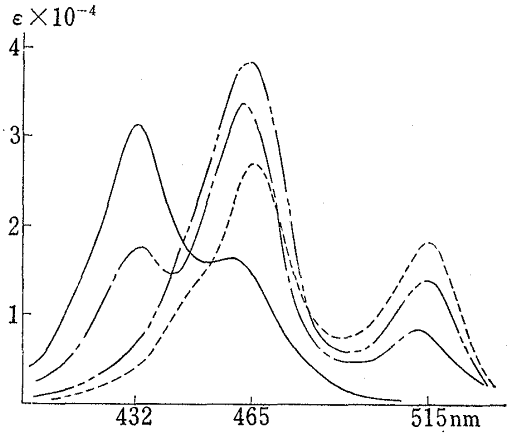

Fig. 1. Absorption Spectra of Estradiol-3-methyl Ether (II) in Sulfuric Acid of Various Concentrations

II: 20 μg
H_2SO_4: 3 ml at 100° for 20 min
———: 97.2% —-—: 75.0%
– – – –: 66.8% -------: 60.0%

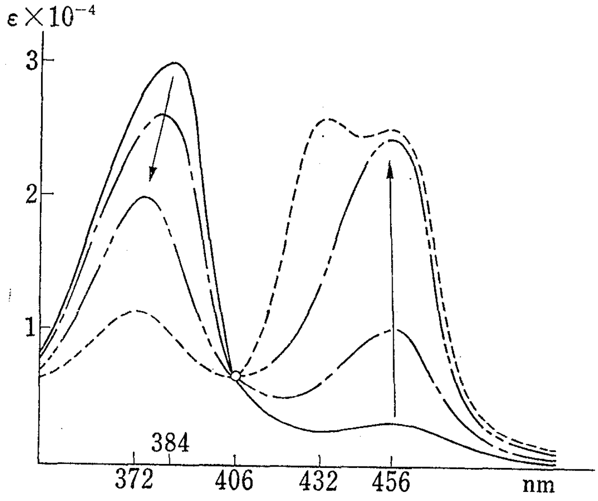

Fig. 2. Absorption Spectra of Estradiol-3-methyl Ether (II) in 97.2% Sulfuric Acid

II: 51 μg
H_2SO_4: 10 ml at 25°
———: 15 min —·—·—: 25 min
— — —: 45 min -------: 16 hr

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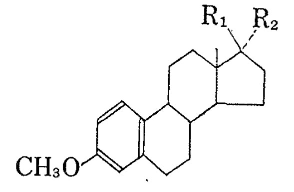
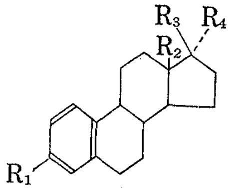
[9] G.A. Olah, *J. Am. Chem. Soc.*, **94**, 808 (1972).

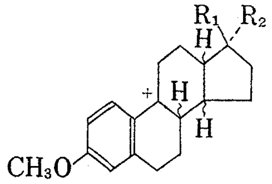
[10] K. Tanabe and T. Takeshita, "Acid-Base Catalysis," Sangyo Tosho Publ. Co., Ltd., Tokyo, 1965, p. 326.



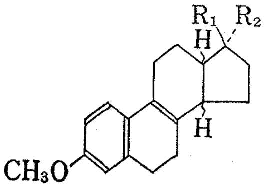
I : R_1, R_2=⟩C=O
II : R_1=OH, R_2=H
III : R_1=H, R_2=OH
IV: R_1=H, Δ^{16}
VI: R_1=OH, R_2=CH_3
XI: R_1=R_2=H



V : R_1=OCH_3, R_3=CH_3, R_4=H, Δ^{13}
VII: R_1=OCH_3, R_3=R_4=CH_3, Δ^{13}
IX: R_1=OH, R_2=CH_3, R_3=H, R_4=OH
X : R_1=H, R_2=R_4=CH_3, R_3=OH



VIIIa : R_1=CH_3, R_2=H
VIIIb : R_1=R_2=CH_3



XIIa : R_1=CH_3, R_2=H
XIIb : R_1=R_2=CH_3

Chart 1

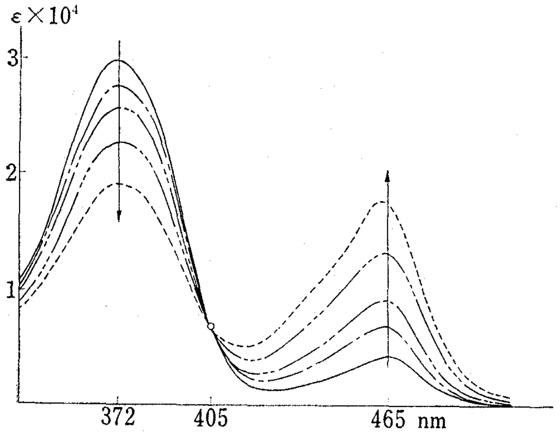


Fig. 3. Increase in Maximum Absorption at 465 nm with Elapse of Time

25.9 μg of 3-methoxy-estra-1,3,5(10),16-tetraene (IV) was dissolved in 4 ml of 97.2% H_2SO_4 followed by diluting with 1 ml of water; all procedures were carried out at about 25°.
———: 30 min, ———: 21 hr, —·—·—: 47 hr, —··—··—: 98 hr, - - - -: 168 hr

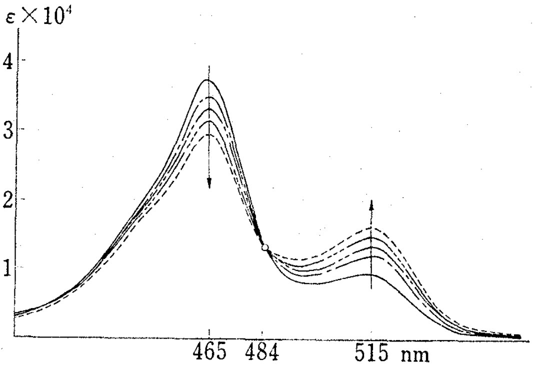
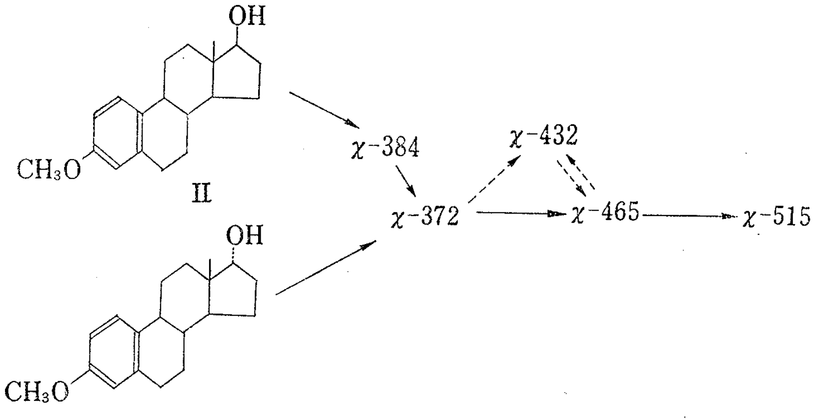


Fig. 4. Increase in Maximum Absorption at 515 nm with Elapse of Time

3 ml of the colored solution prepared for Fig. 3 was diluted with 2 ml of water; all procedures were carried out at about 25°.
———: 5 hr, ———: 72 hr, ———: 96 hr, —··—··—: 125 hr - - - -: 166 hr

was observed to be variable in the range between 456 and 465 nm with the concentration of sulfuric acid. When each of 17α-estradiol methyl ether (3-methoxyestra-1,3,5(10)-trien-17α-ol) (III), 3-methoxyestra-1,3,5(10),16-tetraene (IV), 3-methoxy-17β-methyl-18-norestra-1,3,5(10),13-tetraene (V), 3-methoxy-17α-methylestra-1,3,5(10)-trien-17β-ol (VI), and 17,17-dimethyl-3-methoxy-18-norestra-1,3,5(10),13-tetraene (VII) was dissolved in 97.2% sulfuric acid, the absorption maximum at 372 nm (ε: 33000—34000) appeared immediately, which was steady for long time at room temperature. With decrease in acidity by diluting the reaction mixture with water, the absorption maximum thus appeared was shifted gradually to 465 nm and the further dilution transfered it to 515 nm which is given by the chromophore (abbreviated as χ-515) produced in the second step of the Kober reaction (Fig. 3 and 4). From these results, it may be assumed that the chemical species χ-372 is initially formed in the Kober reaction, which is finally transformed into the species χ-515 through the intermediary

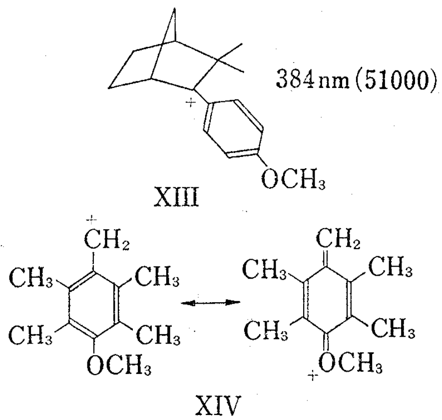


III or IV—VII

Chart 2

species χ-465. Although it remains unknown that χ-432 can be derived from χ-465, formation of the former species is generally observable in the system of highly concentrated sulfuric acid and is more slow than that of the latter. The difference in the fashion of light absorption, which was observed between the reaction of II with 97.2% sulfuric acid and those of the other substrates (III and VI), may be assumed to be due to the velocity of dehydrating the hydroxyl group at C-17.[11]

Since χ-372 is formed immediately from these substrates (III—VII) in 97.2% sulfuric acid, it may be assumed to be the carbocation produced by protonation or dehydration. Bartlett, *et al.*[12] proposed that the maximum absorption at 384 nm shown by *p*-anisylcamphenilol or the corresponding olefin in formic, hydrochloric, or sulfuric acid is due to the carbocation (XIII). Olah, *et al.*[13] found that the characteristic light absorptions at 334 nm and 412—420 nm are due to the cation (XIV) and predicted the red shift of the maxima caused by the increase in the extent of delocalizing the positive charge when the methyl group adjacent to methoxylated carbon is displaced with hydrogen. In nuclear magnetic resonance (NMR)



334nm (24000)
412—420nm (700)

Chart 3

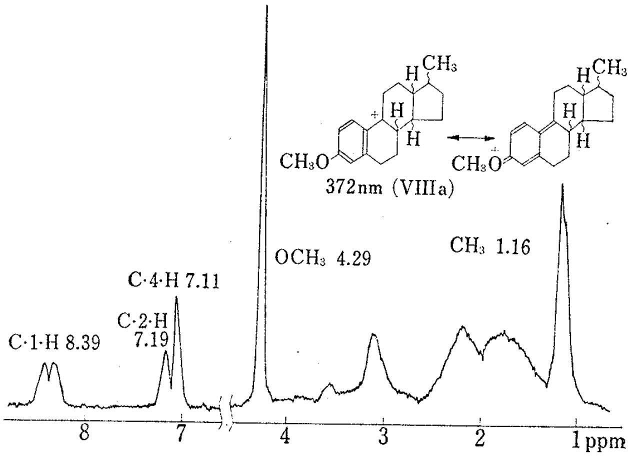


Fig. 5. NMR Spectrum of Carbonium Ions in Concentrated Sulphuric Acid

28 mg of 17β-methyl-3-methoxy-18-norestra-1,3,5(10),13-tetraene (V) in 0.5 ml of 97.2% H_2SO_4. 35°, 60 Mc; (V) in CCl_4: C-1-H (7.13), C-2-H (6.56), C-4-H (6.48), OCH_3 (3.72), CH_3 (1.03).

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11) H. Kägi and K. Miescher, *Helv. Chim. Acta*, 22, 683 (1939); B. J. Boscott, *Nature*, **164**, 140 (1949); T. Lunaas, *Acta Chem. Scand.*, **18**, 321 (1964).

12) P.D. Bartlett, E.R. Webster, C.E. Dills, and H.G. Richey, *Ann*,. **623**, 217 (1959).

13) C.A. Cupas, M.B. Comisarow, and G.A. Olah, *J. Am. Chem. Soc.*, **88**, 361 (1966).

study of the carbocations, it was pointed out that the more is situated a proton nearly to the positive charge, the more is it deshielded and shows a resultant shift of its signal to the field lower than that given by the corresponding conjugate base. Also, the more was facilitated the increase in the extent of delocalizing positive charge by the factors such as resonance, the less was such shift. All of the NMR signals due to III, IV, and V shifted to lower field in 97.2% sulfuric acid, though they kept a similar signal-pattern given by V in carbon tetrachloride (Fig. 5). The shifts of the signals from these standard values due to V were found to be in the following extent (ppm): C_1-H(−1.26)>C_2,C_4-H(−0.63)>OCH_3(−0.57)>CH_3 (−0.13). Since the solvent effect of sulfuric acid has been estimated as about −0.15 ppm,[14] it may thus be assumed that χ-372 is a carbocation (VIII), in which the positive charge is conjugated with the ring A of anisole type; the extent of chemical shifts of VIIIa and VIIIb was highly consistent with that observed in the NMR spectrum of *p*-anisyldimethylcarbenium ion (XV) in SbF_5-SO_2 system at −78°.[15] The conclusion may also be supported by the experimental facts as follows: 1) The sulfuric acid (97.2%) solution of 17α-estradiol (estra-1,3,5(10)-triene-3,17α-diol) (IX) having free hydroxyl group at C_3 and 17α-methylestra-1,3,5(10)-trien-17β-ol (X) without function at the same carbon atom gave absorption maxima at 362 and 348 nm, respectively, showing thus the substituent-effect at C_3 and consequently the conjugation of positive charge with the aromatic ring A in VIII. 2) The same solution of 3-methoxyestra-1,3,5(10)-triene (XI) which is in lower oxidation state than those of II—VII and is deficient in the structural requirement of the positive Kober reaction,[7c,8] also produced χ-372 gradually. Since the rate of its formation was lower than those of II—VII, sulfuric acid was likely to react with XI in an oxidative fashion by abstracting a hydride anion at C_9. In fact, the rate was reduced in the lower concentration of the acid and it was, on the contrary, facilitated by adding some oxidizing agent such as selenic acid (Fig. 6). 3) Most important is that the conjugate base (XII) of VIII was produced in a higher yield of 80—90% when III—VII were subjected to the reaction with 97.2% sulfuric acid at room temperature

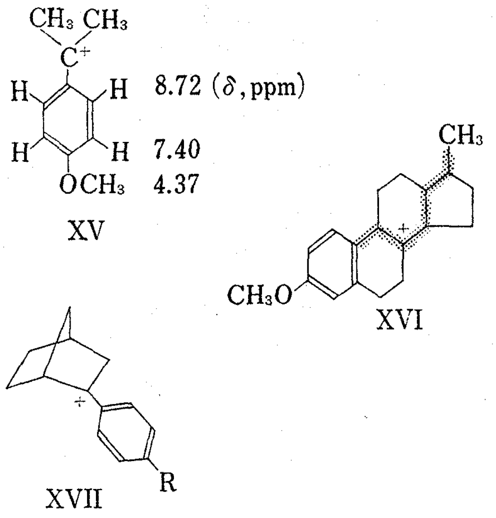

Chart 4

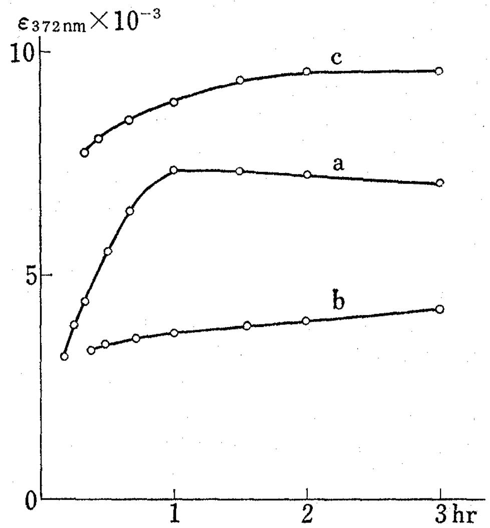

Fig. 6. Formation-rates of χ-372 from 3-Methoxyestratriene (XI) in Sulfuric Acid

XI: 120 μg/5 ml, at 25°
a: 97.2% H_2SO_4
b: 88.0% H_2SO_4
c: 88.0% H_2SO_4-0.01% H_2SeO_4

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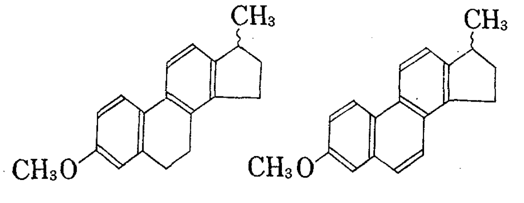
14) J.A. Pople, H.J. Bernstein, and W.G. Schneider, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill, New York, 1950.

15) G.A. Olah, M.B. Comisarow, and C.J. Kim, *J. Am. Chem. Soc.*, 91, 1458 (1969).

for several minutes.[2] From these results, it may be postulated that the carbocation is initially produced through protonation as well as subsequent dehydration during the earlier parts of the Kober reaction of steroidal estrogen and is then transformed into the more stable cation (VIII) through methyl-transfer and hydride-shift.

As to the chemical species responsible for the absorption maxima at 465 and 515 nm, Jones, *et al.*[7c] proposed the nonclassical resonating structure (sigma-delocalization) of the carbonium ion (XVI). However, the dehydrogenated products (XVIII and XIX) were obtained from the substrates (I and II)[1] and the formation of χ-465 from χ-372 was accelerated with the oxidizing agents such as selenic acid and bromine molecules.[16] The cation (XVII) has been proved to be the classical carbenium ion which is stabilized by the phenyl group.[17] Under the conditions of the Kober reaction employed in the analytical use, the cation VIII thus formed may subsequently be oxidized into the characteristic chromophores χ-465 and χ-515, through which the highly dehydrogenated products[1] such as XVIII and XIX may finally be formed. The chemical constitutions of these Kober chromophores are now under investigation.



XVIII XIX

Chart 5

Experimental

Materials

Estradiol (estra-1,3,5(10)-triene-3,17β-diol), estrone (3-hydroxyestra-1,3,5(10)-trien-17one), 17α-estradiol (estra-1,3,5(10)-trien-3,17α-diol) (IX), and desoxyestrone (estra-1,3,5(10)-trien-17-one) were obtained from the commercially available sources and used after recrystallization. The methyl ethers (I,II, and III) were prepared from the respective substrate with diazomethane. 3-Methoxy-17α-methyestra-1,3,5(10)-trien-17β-ol (VI)[18] and 17α-methylestra-1,3,5(10)-trien-17β-ol (X)[19] were prepared from II and desoxyestrone with CH_3MgI, respectively. Preparation of 17,17-dimethyl-3-methoxy-18-norestra-1,3,5(10),13-tetraene (VII), mp 72—74° (lit. 72—75°),[18] was carried out by reaction of VI with 6 N HCl. 3-Methoxyestra-1,3,5(10)-triene (XI), mp 76—78° (lit. 75—76°),[20] was synthesized by Huang-Minlon reduction of II.

3-Methoxyestra-1,3,5(10),16-tetraene (IV)——After a mixture of II (2.20 g), *p*-toluenesulfonylhydrazide (1.92 g), EtOH (120 ml), and AcOH (1 ml) was refluxed for 30 min, the solution was concentrated to half volume and cooled. The precipitates were collected and recrystallized from EtOH yielding *p*-toluenesulfonylhydrazone (2.40 g), mp 203—205° (lit. 201—203°).[21] To a solution of the hydrazone (3.92 g) in ether (200 ml) was added ether solution (50 ml) of *n*-butyl lithium (approximately 0.025 mole) with stirring during 10 min and stirred further for 5 hr. The reaction mixture was diluted with water and extracted with benzene. The organic layer was washed with water, dried over Na_2SO_4, and evaporated *in vacuo* to give an oily residue (1.92 g), recrystallization of which gave a colorless plates (0.72 g), mp 63° (lit. 63°),[20] from MeOH–benzene.

3-Methoxy-17β-methyl-18-norestra-1,3,5(10),13-tetraene (V)——A mixture of metallic Na (1.80 g) and ethylene glycol (75 ml) was stirred and warmed gently to dissolve Na entirely. To the solution was added *p*-toluenesulfonylhydrazone (3.92 g) of II with stirring and the mixture was heated at 130—170° for 60 min. The cooled solution was diluted with water and extracted with benzene. The organic layer was washed with water, dried over Na_2SO_4 and evaporated to dryness yielding a crystalline residue (2.66 g), recrystallization of which gave colorless crystals, mp 109—109.5° (lit. 109—110°),[21] from EtOH.

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[16] Details will be reported later.

[17] G.A. Olah, J.R. DeMember, C.Y. Lui, and A.M. White, *J. Am. Chem. Soc.*, **91**, 3958 (1969); P.v.R. Schleyer, D.C. Kleinfelter, and H.G. Richey, Jr., *ibid.*, **85**, 479 (1963).

[18] J. Torreilles and A. Crastes de Paulet, *Bull. Soc. Chim. France*, **1968**, 4886.

[19] A.H. Goldkamp, W.M. Hoen, R.A. Mikulec, F. Nutting, and D.L. Cook, *J. Med. Chem.*, **8**, 409 (1965).

[20] L. Caglioti and M. Magi, *Tetrahedron*, **19**, 1127 (1963).

[21] W.F. Johns, *J. Org. Chem.*, **26**, 4583 (1961).

Methods

Absorption Spectra——1) Dichloromethane solution of sample was slowly added to 10 ml of conc. H_2SO_4 (97.2%) with rapid stirring at 0—5° and the acid layer was measured on a Hitachi EPS-3T spectrophotometer at 25°.

2) To a dried sample was added 10 ml of conc. H_2SO_4 at room temperature. The solution was shaked vigorously to be homogeneous and measured.

No appreciable difference in absorption spectra was detected between these procedures.

NMR Spectra of χ-372——To each sample (III—VII) (20—30 mg) was added conc. H_2SO_4 with vigorous shaking. The solution became homogeneous and slightly yellow. NMR spectra were recorded on a Hitachi Model R-20-B spectrometer with external capillary 10% tetramethylsilane in CCl_4 as a reference at 35°. No spectral change was detected in each sample when it was diluted with conc. H_2SO_4 after NMR measurement.