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Chromogenic Reactions of Steroids with Strong Acids. VIII.[1] Mechanism of the First Stage in the Kober Reaction

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Dissolution of 3-methoxy-17β-methyl-18-norestra-1,3,5(10),13-tetraëne into 77.3% sulfuric acid gave the chromophore χ-372 (I) which changed gradually to χ-465 (II). This change was faster and its acceleration with oxidant was greater in the systems of lower acid strength. In 97.2% sulfuric acid, 3-methoxyestra-1,3,5(10),9(11)-tetraën-17-one and 3-methoxyestra-1,3,5(10),8,14-pentaën-17-one showed maximum absorptions at 363 and 467 nm, respectively, and were then recovered almost quantitatively from the solution. Thus, the conjugate base of I was assumed to give II through an oxidative process.

13ξ,14ξ-Epoxy-3-methoxy-17β-methyl-18-norestra-1,3,5(10)-triëne (III) immediately showed a stable maximum absorption at 465 nm in 97.2% sulfuric acid, from which the estrapentaëne (IV) was then isolated. Compared with the chemical shifts of I, NMR spectra of III as well as IV in sulfuric acid suggested II to be the carbocation.

The same experiments were also made on related compounds and corresponding results were obtained. Consequently, it is proposed, for the mechanism of the Kober reaction in its first stage, that the conjugate base of the initially formed carbocation χ-372 (I) is oxidized by sulfuric acid into the chromophoric cation χ-465 (II).

Keywords——color reaction; estrogen; Kober reaction; NMR; oxidation; steroidal carbocation; sulfuric acid

The urinary excretion of estriol is an important parameter for assessing the feto-placental function and thus for monitoring the course of pregnancy.[3] Since Kober[4] found that sulfuric acid gave a color reaction highly specific for steroidal estrogens, several modifications[5] have been utilized for the determination of urinary estrogens in clinical chemistry. The Kober reaction consists of two stages of color development; the maximum absorption at 465 nm appears in the first stage of the reaction and then a more bathochromic absorption occurs at 515 nm in the second stage. The colorimetric determination of estrogens is usually based on the latter absorption in the Kober reaction. In order to elucidate the mechanism of this reaction, we have studied the structure of the products in the reaction of estrogens with sulfuric acid,[6] specificity of the Kober reaction,[7] and the mechanism of this reaction in its earlier period.[1,8] It was clarified from these studies that the carbocation[9] (Ia) is formed at the beginning of the Kober reaction by removal of the hydroxyl group at C(17)followed by the Wagner-Meerwein rearrangement of the methyl group from C(13)to C(17), and hydride shifts. We show in this paper that the chromophoric species ($\lambda_{\max}$ 465

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1) Part VII: M. Kimura and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **24**, 181 (1976).
2) Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*
3) V. Goebelsmann, K. Sjöberg, N. Wquist, and E. Diczfalusy, *Endocrinol.*, **76**, 660 (1965).
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5) *a*) J.B. Brown, *J. Endocrinol.*, **8**, 196 (1952); *b*) G. Ittrich, *Z. Physiol. Chem.*, **312**, 1 (1958); *c*) J.R.K. Preedy, "Methods in Hormone Research," Vol. 1, ed. by R.I. Dorfman, Academic Press, London, 1962, p. 1; *d*) Y. Osawa and W.R. Slaunwhite, Jr., *Steroids*, **15**, 73 (1970).
6) M. Kimura, M. Kawata, K. Akiyama, K. Harita, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **21**, 1741 (1973).
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8) M. Kimura, K. Akiyama, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **22**, 643 (1974).
9) G.A. Olah, *J. Am. Chem. Soc.*, **94**, 808 (1972).

nm) appearing in the first stage of the Kober reaction is the carbocation (IIa) and that the conjugate base (VIIa) of the cation Ia (λ_{max} 372 nm) is oxidized by sulfuric acid into the chromophore (IIa) having a longer conjugated system.

Results and Discussion

The chromophoric χ-465 (designation for the species indicating a maximum absorption at 465 nm) has previously been reported to be produced from χ-372 (Ia).[8] Detailed chemistry pertaining to this transformation had to be elucidated in order to obtain a clue to the chemical structure of χ-465. When 3-methoxyestra-1,3,5(10), 16-tetraëne (III) was dissolved in concentrated sulfuric acid, the chromophoric χ-372 (Ia) appeared immediately and was transformed gradually into χ-465 on dilution of the acidic solution with water.[8] Although it can be predicted that decrease in acid strength labilizes the carbocation and

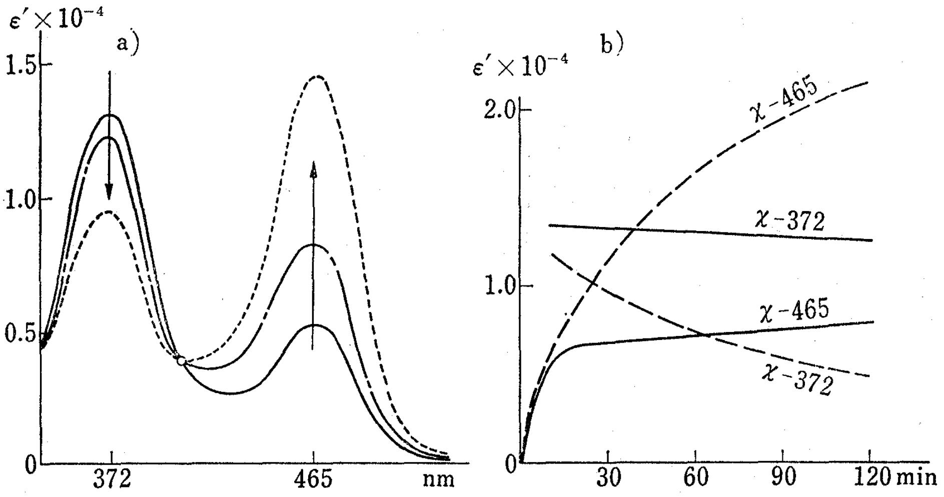


Fig. 1. Effect of Oxidant (H_2SeO_4) on the Conversion of χ-372 into χ-465

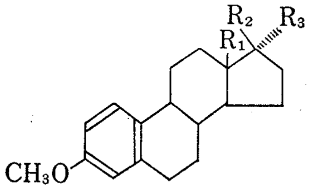
———: 12 min ———: in the presence of H_2SeO_4 (1.5 mg)
— — —: 4 hr
⋯⋯⋯: 27 hr
60 μg of VIa was dissolved in 5 ml of 77.3% H_2SO_4 at 25°
ε': apparent molar extinction coefficient

Table I. Effect of Acid Strength and Oxidant (H_2SeO_4) on the Conversion of χ-372 into χ-465

Concentration of sulfuric acid (H_0)	$\frac{H_2SeO_4}{IVa}$ (molar ratio)	$t_{3/4}$ of E at 372 nm (hr)
97.2% H_2SO_4	0	>200
(−10.25)	1	>200
	2	>200
	45	*ca.* 150
88.0% H_2SO_4	0	125
(−8.61)	1	90
	2	70
	45	23
77.3% H_2SO_4	0	22
(−6.92)	1	16
	2	10
	4	3.5
	16	1.5
	45	0.5

leads to its secondary change, the oxidative effect of such acid that can play as an oxidant should also be examined with respect to this change. In fact, oxidation was found to occur in the Kober reaction of estrone methyl ether (IV) and estradiol methyl ether (Va), from the analysis of their products.[6] Leftin[10] reported that the absorption at about 300 nm shown by monoölefins in sulfuric acid is due not to alkyl cations formed by the protonation, but to alkenyl cations produced by the simultaneous oxidation with the acid as an oxidant, and that the reaction is accelerated by selenic acid which is a more potent oxidant. According to the method employed by Leftin, the experiments were designed so as to examine the acidic as well as the oxidative effect of sulfuric acid on the reaction as follows:

Dissolution of 3-methoxy-17*β*-methyl-18-norestra-1,3,5(10),13-tetraëne (VIa) into 77.3% sulfuric acid gave χ-372 which changed gradually to χ-465 and this change was accelerated by the addition of selenic acid (Fig. 1) or oxidants such as bromine, ferricyanate and *p*-benzoquinone. The acidic as well as oxidative effect of these acids on this change is summarized in Table I where $t_{3/4}$ of $E_{372\,\mathrm{nm}}$ represents the time required for the absorbance at 372 nm to be reduced to 3/4 of its initial value. This table reveals that the change of χ-372 to χ-465 is fast and the acceleration of this change by an oxidant is great in the



III : $R_1=CH_3$, $R_2=H$, Δ^{16}
IV : $R_1=CH_3$, $R_2,R_3=$ >C=O
VIa: $R_2=CH_3$, $R_3=H$, Δ^{13}
VIb: $R_2=R_3=CH_3$, Δ^{13}
XIIIa: $R_2=CH_3$, $R_3=H$, 13,14-epoxide ($\alpha+\beta$)
XIIIb: $R_2=R_3=CH_3$, 13,14-epoxide ($\alpha+\beta$)

Chart 1

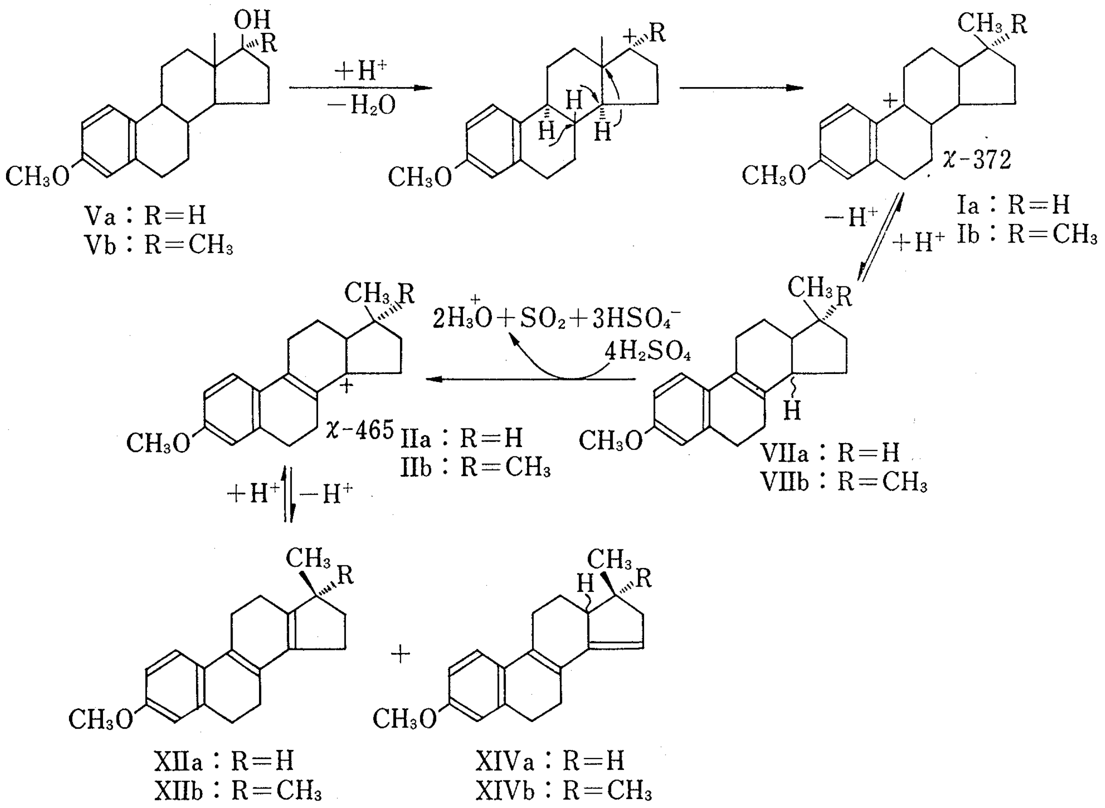


Chart 2

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10) H.P. Leftin, "Carbonium Ions," Vol. 1, ed. by G.A. Olah and P.v.R. Schleyer, Wiley Interscience, New York, N.Y., 1968, p. 383.

systems of the lower acid strength. This change was reasonably assumed to proceed through the conjugate base (VIIa) of χ-372 (Chart 2) in such a way that the acid strength is responsible for an equilibrium between χ-372 and VIIa, on the one hand, and the oxidative potentiality participates in transforming VIIa to χ-465, on the other. In spite of the higher oxidative potentiality of 97.2% sulfuric acid, the equilibrium shifted to the side of χ-372 owing to the higher acid strength of the solution and the resultant stabilization of χ-372 prevented its change to χ-465, and also the oxidative effect of selenic acid. On the contrary, decrease in concentration of the acid accelerated the change, suggesting an increase in concentration of VIIa which should subsequently be oxidized into χ-465. Since these results were in good agreement with those obtained by Leftin, it was probable that χ-465 is the carbocation (IIa) which is formed through the elimination of a hydride from C(14) of VIIa (Chart 2). Dissolution of 3-methoxyestra-1,3,5(10),9(11)-tetraën-17-one (VIII) and 3-methoxyestra-1,3,5(10),8,14-pentaën-17-one (IX) in concentrated sulfuric acid immediately gave maximum absorptions at 363 nm (ε 35000) and 467 nm (ε 50000), respectively (Fig. 2). Nuclear magnetic resonance (NMR) spectra (Fig. 3 and 4) of these acidic solutions suggested the presence of carbocations such as X and XI, which was also suggested by the fact that the starting materials (VIII and IX) were recovered almost quantitatively from these colored solutions. From these results and from a consideration of the relationship between structures and wavelengths at the maximum absorptions of these carbocations, it may be reasonable to assume that χ-465 is the cation (II). Thus, the estrapentaëne (XII), which is the conjugate base of II, should then be protonated in concentrated sulfuric acid to give immediately the maximum absorption at 465 nm, as indicated below.

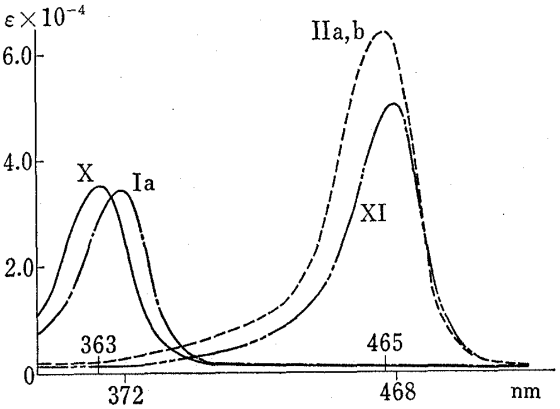

Fig. 2. Absorption Spectra of Steroidal Carbocations in Concentrated Sulfuric Acid at 25°

steroid: 15—30 μg 97.2% H_2SO_4: 5 ml

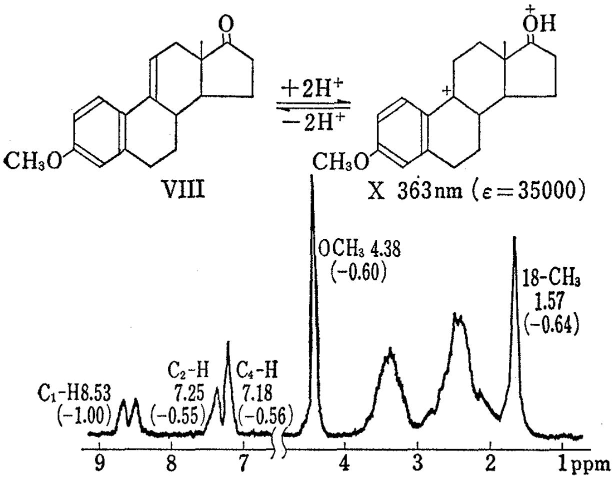

Fig. 3. NMR Spectrum of VIII in Concentrated Sulfuric Acid at 60 Mc

30 mg of VIII in 0.5 ml of 97.2% H_2SO_4, 35° part per million from external (capillary) tetramethylsilane difference in ppm from parent compound in parentheses

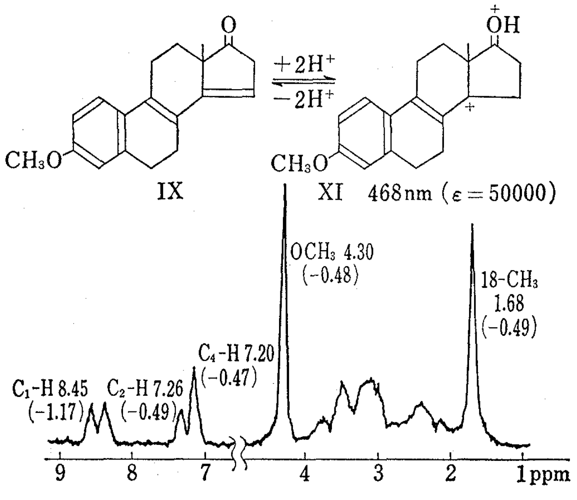

Fig. 4. NMR Spectrum of IX in Concentrated Sulfuric Acid at 60 Mc

30 mg of IX in 0.6 ml of 97.2% H_2SO_4, 35°, part per million from external (capillary) tetramethylsilane, difference in ppm from parent compound in parentheses

On treatment with BF_3, 17,17-dimethyl-13ξ,14ξ-epoxy-3-methoxy-18-norestra-1,3,5-(10)-triëne (XIIIb) gives a mixture of the estrapentaëne (XIIb) and its isomers.[11] When XIII as the postulated precursor of the carbocation (II) was dissolved in concentrated sulfuric acid, the stable maximum absorption at 465 nm (ε 64000) was observed as expected (Fig. 2). NMR spectrum of this acidic solution exhibited signals at 8.26 (d, $J=9$ cps), 7.15 (d, $J=9$ cps), 7.07, 4.19, 1.33, and 0.94 ppm assignable to the protons of C(1)-H, C(2)-H, C(4)-H, C(3)-OCH_3, C(17)β-CH_3, and C(17)α-CH_3 (Fig. 5). Compared with the chemical shifts of χ-372 (Ia), the signals due to aromatic protons and C(17)β-CH_3 shifted to the higher and lower fields, respectively (Table II). These results and the increase in the relative area of methine and methylene signals in the 2.60—3.50 ppm region to those of Ia in 1.50—2.60 region may suggest that χ-465 is the carbocation (II) whose positive charge is delocalized along a more extended conjugation system. The triëne (XIIIb) was dissolved in concentrated sulfuric acid during 5 min at room temperature and the colored solution was poured into ice water and extracted with benzene. The extracts gave a mixture of the conjugate bases of the carbocation (II) as colorless prisms, mp 98—102°, λ_{max} 313 nm (ε 17400), *m/e* 280 (M^+), $C_{20}H_{24}O$, in 80% yield. Since mass (MS) spectrum (Fig. 6) gave a base peak of *m/e* 265 (M^+—15), the major component of the mixture thus obtained was considered to be the pentaëne (XIIb) which can easily eliminate an allylic methyl group giving such a fragment. NMR spectrum (in $CDCl_3$) of the mixture gave singlet signals due to methyl group at 0.88 and 1.16 ppm having an integration by a factor of 1/3 to that of *gem*-C(17)-dimethyl

Table II. Comparison of PMR Chemical Shifts[a] of χ-372 and χ-465

	C_1-H	C_2-H	C_4-H	OCH_3	17β-CH_3	17α-CH_3
χ-372 (Ia)	8.39	7.19	7.11	4.29	1.16	—
χ-465 (IIb)	8.26	7.15	7.07	4.19	1.33	0.94
Δppm	0.13	0.04	0.04	0.10	−0.17	—

a) part per million relative to te tramethylsilane in 97.2% H_2SO_4 at 35°, 60 Mc

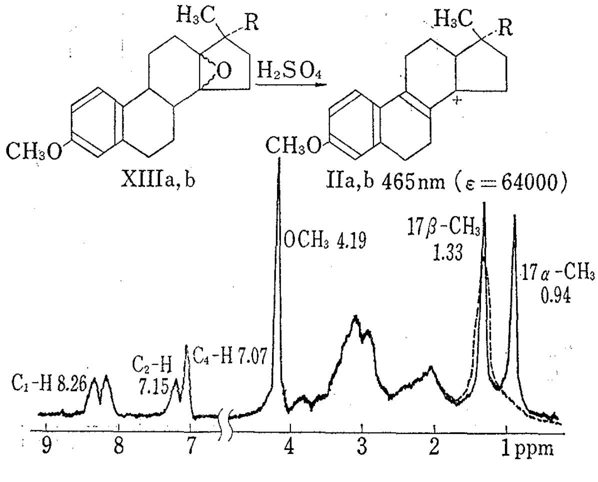

Fig. 5. NMR Spectra of χ-465 in Concentrated Sulfuric Acid at 60 Mc

38 mg of epoxide in 0.5 ml of 97.2% H_2SO_4, 35°, part per million from external (capillary) tetramethylsilane
——: IIa ——: IIb

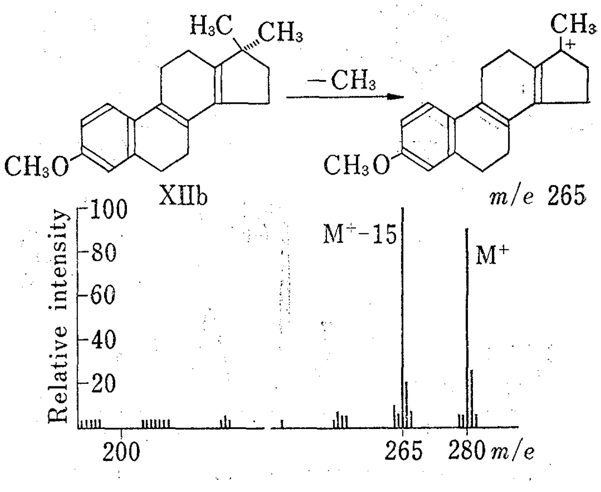

Fig. 6. Mass Spectrum of (XIIb+XIVb)

source 200°, 79 eV

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

groups of XIIb at 1.06 ppm. Since the spectrum also gave a broad singlet signal (1/4 ^{1}H) due to C(15)-vinyl proton at 5.02 ppm, the crystalline mixture was found to contain the isomeric pentaëne (XIVb), and consisted of XIIb and XIVb in 3: 1 ratio. Similarly, the conjugate bases (XIIa and XIVa, 3: 1) of IIa were obtained in about 35 % yield when 13ξ,14ξ-epoxy-3-methoxy-17β-methyl-18-norestra-1,3,5(10)-triëne (XIIIa) was dissolved in concentrated sulfuric acid. The mixture of XIIa and XIVa, as well as that of XIIb and XIVb, thus obtained gave the light absorption and NMR spectra almost identical with those of the epoxides (XIIIa and XIIIb) when they were treated with concentrated sulfuric acid.

These results indicate that the chromophoric species χ-465 appearing in the first stage of the Kober reaction is the carbocation (IIa) which is produced by sulfuric acid through an oxidative process of the conjugate base (VIIa) of χ-372 (Ia) initially formed in the earlier period of the reaction.

Experimental[12]

Materials——3-Methoxy-17β-methyl-18-norestra-1,3,5(10),13-tetraëne (VIa) and 17,17-dimethyl-3-methoxy-18-norestra-1,3,5(10),13-tetraëne (VIb) were prepared as described in the previous report.[8] *dl*-3-Methoxyestra-1,3,5(10),8,14-pentaën-17-one (IX) was kindly supplied by Dr. T. Miki.

3-Methoxyestra-1,3,5(10),9(11)-tetraën-17-one (VIII)——To a solution of IV (500 mg) in dioxane (30 ml) was added dropwise a solution of 2,3-dichloro-5,6-dicyanobenzoquinone (1.0 g) in dioxane (20 ml) and stirred for 15 min at room temperature. After removal of the precipitate by filtration the filtrate was evaporated *in vacuo* and the residue obtained was dissolved in benzene. The organic layer was washed with 10% NaOH and water and dried over anhydrous Na_2SO_4. Upon usual work-up the residue (440 mg) obtained was submitted to preparative TLC using benzene–EtOH (95: 5) as developing solvent. Elution of the adsorbent corresponding to the spot ($Rf=0.51$) gave a 1: 1 mixture of the starting material and the desired substance (VIII) as colorless prisms (360 mg). The prisms were dissolved in conc. H_2SO_4 (20 ml) and stirred for 20 min at room temperature. In this reaction, IV was converted into the hydrophilic compound through C_2-sulfonation.[1] On the other hand, sulfonation did not occur in VIII because of lowering electron density of A-aromatic ring accompanied by the rapid formation of stable carbocation (X).

The resulting solution was dropped into vigorously stirred ice-water and extracted with benzene. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness yielding a crystalline residue. Recrystallization of the residue from MeOH gave VIII (140 mg) as colorless prisms. mp 146—148° (lit.[13] 147—148°). Mass Spectrum *m/e*: 282 (M^+). UV λ_{max}^{EtOH} nm (ε): 264 (21000). *Anal.* Calcd. for $C_{19}H_{22}O_2$: C, 80.81; H, 7.85. Found: C, 80.72; H, 7.69. NMR spectrum (in $CDCl_3$) δ: 0.94 (3H, s, CH_3), 3.79 (3H, s, OCH_3), 6.13 (1H, broad s, C_{11}–H), 6.60 (1H, d, $J=2.5$ cps, C_4–H), 6.68 (1H, q, $J_1=9$ cps, $J_2=2.5$ cps, C_2–H), 7.52 (1H, d, $J=9$ cps, C_1–H). $[\alpha]_D^{20}=+292.0°$ ($c=0.432$, $CHCl_3$).

13ξ,14ξ-Epoxy-3-methoxy-17β-methyl-18-norestral-1,3,5(10)-triëne (XIIIa)——A mixture of 343 mg of VIa, 320 mg of *m*-chloroperbenzoic acid, 6 ml of 0.5 M $NaHCO_3$ and 10 ml of CH_2Cl_2 was stirred for 4 hr at room temperature under nitrogen atmosphere. The resulting solution was diluted with 5% Na_2SO_3 and extracted with benzene. The organic layer was washed with 5% Na_2SO_3, 5% $NaHCO_3$ and water successively and dried over anhydrous Na_2SO_4. After usual work-up the residue obtained was submitted to the preparative TLC using benzene–EtOH (95: 5) as developing solvent. The adsorbent corresponding to the spot ($Rf=0.58$) was eluted with $CHCl_3$, and the eluate was crystallized from MeOH to give XIIIa (170 mg) as colorless leaflets. mp 125—129°. Mass Spectrum *m/e*: 284 (M^+). *Anal.* Calcd. for $C_{19}H_{24}O_2$: C, 80.24; H, 8.51. Found: C, 80.42; H, 8.43. NMR (in $CDCl_3$) δ: 0.95 (3H, d, $J=6$ cps, CH_3 of α- or β-epoxide), 1.07 (3H, d, $J=6$ cps, CH_3 of α- or β-epoxide), 3.70 (3H, s, OCH_3), 6.55—7.25 (3H, arom.).

17,17-Dimethyl-13ξ,14ξ-epoxy-3-methoxy-18-norestra-1,3,5(10)-triëne (XIIIb)——A mixture of 485 mg of VIb, 395 mg of *m*-chloroperbenzoic acid, 6 ml of 0.5 M $NaHCO_3$ and 17 ml of CH_2Cl_2 was stirred for 17 hr at room temperature under nitrogen atmosphere. The resulting solution was diluted with 5% Na_2SO_3 and extracted with benzene. The organic layer was washed with 5% $NaHCO_3$ and worked up as usual. The

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[12] All melting points were taken on a micro hot-stage apparatus and are uncorrected. For the preparative TLC silica gel (Wakogel B-5-F) was used as an adsorbent. Ultraviolet and visible spectra were measured by Hitachi Model 3T recording spectrometer. NMR spectra were recorded on Hitachi MOdel R-20-B spectrometer at 60 Mc using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, and q=quartet. Mass spectral measurement was run on Hitachi Model RMU-6R spectrometer.

[13] R.C. Cambie and V.F. Carlisle, *J. Chem. Soc.* (*C*), **1970**, 1706.

crude product thus obtained was recrystallized from MeOH to give XIIIb (380 mg) as colorless prisms, mp 99—102°. Mass Spectrum *m/e*: 298 (M+). *Anal.* Calcd. for $C_{20}H_{26}O_2$: C, 80.49; H, 8.78. Found: C, 80.55; H, 8.72. NMR (in $CDCl_3$) δ: 0.95 (6H, s, CH_3 of α- or β-epoxide), 1.07 (6H, s, CH_3 of α- or β-epoxide), 3.72 (3H, s, OCH_3), 6.55—7.25 (3H, arom.).

Reaction of XIIIa with Concentrated Sulfuric Acid——A solution of XIIIb (110 mg) in EtOH (2 ml) was added in portions during 5 min to conc. H_2SO_4 (20 ml) at room temperature with vigorously stirring. The resulting orange colored solution was dropped into vigorously stirred ice-water and then extracted with ether. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness yielding pale yellow crystals (85 mg). Recrystallization of the crystals from EtOH–H_2O gave a 3: 1 mixture of XIIb and XIVb as colorless prisms (60 mg), mp 98—102°. Mass Spectrum *m/e*: 280 (M^+), 265 (M^+-CH_3). UV $\lambda_{\max}^{\text{EtOH}}$: 313 nm ($\varepsilon$=17400). *Anal.* Calcd. for $C_{20}H_{24}O$: C, 85.66; H, 8.63. Found: C, 85.77; H, 8.42. NMR (in $CDCl_3$) δ: 1.16 (6H, s, C_{17}–CH_3 of 13α- or 13β-isomer of XIVb), 1.06 (6H, s, C_{17}–CH_3 of XIIb), 0.88 (6H, s, C_{17}–CH_3 of 13α- or 13β-isomer of XIVb), 3.75 (3H, s, OCH_3), 5.52 (1H, broad s, C_{15}–H of XIVb), 6.60—7.30 (3H, arom.).

Reaction of XIIIa with Concentrated Sulfuric Acid——A solution of XIIIa (110 mg) in EtOH (2 ml) was added in portions during 5 min to conc. H_2SO_4 (20 ml) at room temperature with vigorous stirring. The resulting orange colored solution was dropped into vigorously stirred ice-water and then extracted with ether. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The yellow oil (56 mg) obtained was submitted to TLC using benzene–EtOH (95: 5) as developing solvent. The adsorbent corresponding to the spot (*Rf*=0.70) was eluted with $CHCl_3$ to give a 3: 1 mixture of XIIa and XIVa (38 mg) as a colorless oil. Mass Spectrum *m/e*: 266 (M^+). UV $\lambda_{\max}^{\text{EtOH}}$: 313 nm ($\varepsilon$=16700). *Anal.* Calcd. for $C_{19}H_{22}O$: C, 85.67; H, 8.33. Found: C, 85.85; H, 8.39. NMR (in $CDCl_3$) δ: 0.88 (3H, d, J=6 cps, $C_{17}\beta$–CH_3 of 13α- or 13β-isomer of XIVa), 1.06 (3H, d, J=6 cps, $C_{17}\beta$-CH_3 of XIIa), 1.16 (3H, d, J=6 cps, $C_{17}\beta$–CH_3 of 13α- or 13β-isomer of XIVa), 3.78 (3H, s, OCH_3), 5.58 (1H, broad s, C_{15}–H of XIVa), 6.60—7.30 (3H, arom.).

Absorption Spectra——To a dried sample was added 5 ml of H_2SO_4 at room temperature. The solution was shaken vigorously to be homogeneous and measured on Hitachi EPS-3T spectrometer at 25°.

NMR Spectra of Steroidal Carbocations in Concentrated Sulfuric Acid——To each (30—40 mg) of the samples (VIII, IX, XIIIa and XIIIb) was added 97.2% H_2SO_4 and the mixture was shaken vigorously to be homogenous. NMR spectra were recorded with external capillary tetramethylsilane 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. The starting material which was confirmed by mp as well as NMR spectrum was recovered quantitatively after the measurement, in the cases of VIII and IX.

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