Chromogenic Reactions of Steroids with Strong Acids. X.[1] Behavior of Testosterone in Concentrated Sulfuric Acid

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The behavior of testosterone (I) and its related compounds such as 17-methyl-18-norandrosta-4,13-dien-3-one (II), 17-methyl-18-norandrosta-4,13(17)-dien-3-one (III), androst-4-en-3-one (IV), 5α-androstan-17β-ol (VI), 13ξ,14ξ-epoxy-17-methyl-18-norandrost-4-en-3-one (VIII), and 13ξ,17ξ-epoxy-17-methyl-18-norandrost-4-en-3-one (IX) in concentrated sulfuric acid was studied, in order to elucidate the mechanism of the early stage in the chromogenic reaction of I with sulfuric acid. When I was dissolved into 97.2% sulfuric acid, the chromophoric species was produced indicating a maximum absorption at about 300 nm. From the results of absorption and NMR spectroscopic studies and from the fact that the chromogenic reaction was accelerated by selenic acid as an oxidant, the species was proposed to be the dication (X) which is produced by the protonation, dehydration, angular methyl migration, and oxidation processes and retains the alkenyl as well as hydroxyalkenyl cation chromophores.

Keywords——testosterone; sulfuric acid; selenic acid; alkenyl cation; hydroxyalkenyl cation; dication; chromogenic reaction; NMR

Various chromogenic reactions of steroids with strong acids have widely been utilized for the qualitative and quantitative analyses of steroids. The well known examples of these reactions are Liebermann–Burchard reaction[3] for cholesterol and its modification by Zak et al.,[4] Kober reaction[5] for estrogens, Hammarsten–Yamasaki reaction[6] for bile acids, and Porter–Silber reaction[7] for corticoids. In spite of widespread use of the colorimetric methods for the assay of steroidal compounds in body fluids, the detailed chemistry of these principal reactions have remained obscure.

We have investigated the behavior of steroidal compounds such as estrogens[1,8–12] and testosterone (17β-hydroxyandrost-4-en-3-one; I)[13a–c] in strong acids. In the chromogenic reactions of I with perchloric acid in dichloromethane[13a] and of epitestosterone (17α-hydroxyandrost-4-en-3-one) with sulfuric acid containing bromine in acetic acid,[13b] 17-methyl-18-norandrosta-4,13-dien-3-one (II)[14] and 17-methyl-18-norandrosta-4,13(17)-dien-3-one (III)[15]

1) Part IX: T. Miura and M. Kimura, *Chem. Pharm. Bull.* (Tokyo), **26**, 171 (1978).
2) Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*
3) C. Liebermann, *Chem. Ber.*, **18**, 1803 (1885); H. Burchard, *Chem. Zentralbl.*, **61**, 25 (1890).
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7) C.C. Porter and R.H. Silber, *J. Biol. Chem.*, **185**, 201 (1950).
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9) M. Kimura, M. Kawata, K. Akiyama, K. Harita, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **21**, 1741 (1973).
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13) a) M. Kimura and K. Harita, *Chem. Pharm. Bull.* (Tokyo), **21**, 1205 (1973); b) *Idem, ibid.*, **21**, 1235 (1973); c) *Idem, ibid.*, **21**, 1261 (1973).
14) L.H. Knox, E. Velarde, S. Berger, D. Cuardriello, and A.D. Cross, *J. Org. Chem.*, **29**, 2187 (1964).
15) F. Sondheimer, O. Mancera, M. Urquiza, and G. Rosenkranz, *J. Am. Chem. Soc.*, **77**, 4145 (1955).

were isolated (Chart 1), which are assumed to be the intermediates in these chromogenic reaction. For the measurement of I, various chromogenic reactions with these strong acids have been applied to the colorimetric methods.[16] In all cases of these reactions, two main chromophoric species, X-484 and X-600 (designation for the species indicating a maximum absorption at 484 and 600 nm, respectively; the wave-lengths of the maximum absorption slightly differ depending on the acid systems employed) were produced.

Formation of steroidal carbocations by protonation and/or dehydration can be assumed to be the primary process in the chromogenic reactions of steroidal olefins and alcohols with strong acids. Stability of the carbocations thus formed depends, in general, on the acid strength of the system employed and the unstable carbocations undergo secondary reactions such as rearrangement, oxidation, disproportionation, and polymerization. It was, therefore, important to study first the primary process for the elucidation of the chromogenic reaction mechanism. Since heating and/or lowering the acid strength is/are involved in most of these colorimetric methods, the reaction systems are not appropriate for investigation of the primary process. In concentrated sulfuric acid, the carbocations probably produced at the earlier stage are considered to be comparatively stable. In this paper, the behavior of I and its related compounds in concentrated sulfuric acid is reported and the mechanism of affording the carbocation χ-300 in the early stage of this reaction is proposed.

Results and Discussion

When testosterone (I) or the Δ^{13}-olefin (a 1:1 mixture of II and III) was dissolved into 97.2% sulfuric acid, a maximum absorption at 300 nm appeared immediately as shown in
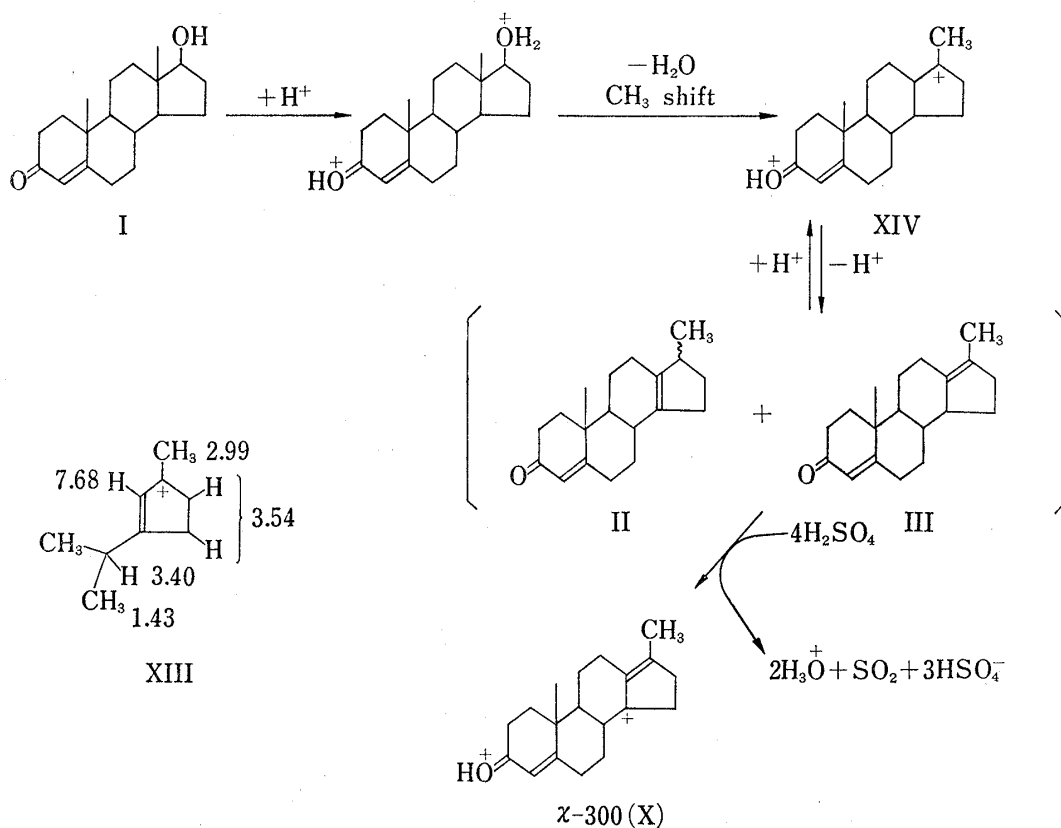

Chart 1. The Primary Process of the Chromogenic Reaction of Testosterone(I) with Sulfuric Acid

16) M.M. Graff, J.T. McElroy, and A.L. Mooney, *J. Biol. Chem.*, **195**, 351 (1952); P.R. Lewis and M.C. Lobban, *J. Histochem. Cytochem.*, **9**, 2 (1961); M. Hosoi, *Yakugaku Zasshi*, **75**, 1506 (1955); J.D. Few, *Analyst*, **90**, 134 (1965).

Fig. 1. Its apparent molar extinction coefficient (ε') was 26000 which was steady for a long time at room temperature. On the other hand, dissolution of androst-4-en-3-one (IV) into the same acid showed a maximum at 295 nm with ε' of 13000. The absorption at 295 nm has proved to be due to the hydroxyalkenyl cations (V) formed from the corresponding α,β-unsaturated ketones.[17] It was noteworthy, in this respect, that the absorption intensity of the acid solution of I or the olefin (II+III) was larger than that of IV. Since the solution of 5α-androstan-17β-ol (VI) in the same acid showed a maximum absorption at 310 nm ($\varepsilon'=$ 4500) as shown in Fig. 1, the reaction initiated by the hydroxyl function or the double bond in the ring D of I or the olefin (II+III), respectively was likely to give another chromophore contributing the absorption at 300 nm, in addition to the hydroxyalkenyl cation (V) chromophore showing the one at 295 nm.
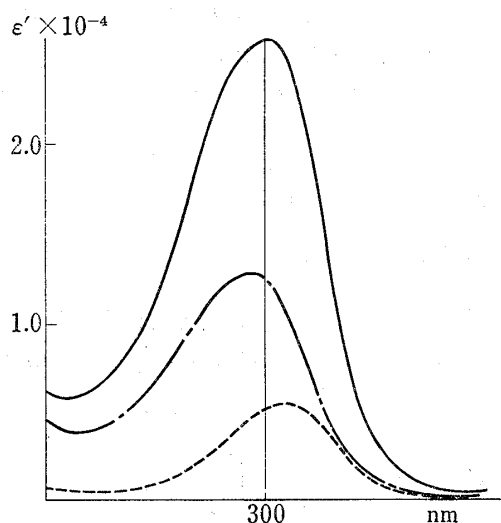

Fig. 1. Absorption Spectra of Testosterone (I) and Related Compounds in Concentrated Sulfuric Acid

Steroid: 50—100μg, 97.2% H_2SO_4: 5 ml.
——: I, II+III, —·—: IV, -----: VI
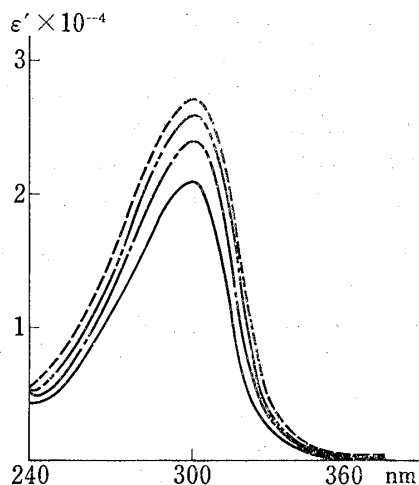ε': apparent molar extinction coefficient.

Fig. 2. Effect of Selenic Acid on the Formation of the Chromophoric χ-300(X)

Steroid: 50 μg, H_2SO_4: 4 ml.
——: II+III in 85.0% H_2SO_4.
-----: II+III in 85.0% H_2SO_4 with 0.2% H_2SeO_4.
—·—: II+III in 85.0% H_2SO_4 with 1.0% H_2SeO_4.
------: VIII+IX in 85.0% or 97.2% H_2SO_4.

Leftin reported that the absorption at about 300 nm shown by monoolefins and alcohols in concentrated sulfuric acid is due not to the alkyl cations formed by protonation and/or dehydration but to the alkenyl cations (VII) produced by simultaneous oxidation with the acid as an oxidant, and that the reaction is accelerated by selenic acid which is a more potent oxidant.[18] As shown in Fig. 2, dissolution of the olefin (II+III) into 85.0% sulfuric acid caused a maximum absorption at 300 nm with ε' of 21000 and, as expected, an increase in its absorption intensity was observed when the same acid was used with selenic acid. Participation of the oxidative process was, therefore, indicated in the formation of a chromophore contributing to the absorption at about 300 nm. An epoxide has been known to form an alkenyl cation or its conjugate base, the corresponding diene, in acidic medium.[12,19] Dissolution of the epoxide, a 1:1 mixture of 13ξ,14ξ-epoxy-17-methyl-18-norandrost-4-en-3-one (VIII) and 13ξ,17ξ-epoxy-17-methyl-18-norandrost-4-en-3-one (IX), into 97.2% or 85.0% sulfuric acid gave the absorption spectrum ($\lambda_{max}=300$ nm, $\varepsilon'=27000$) similar to that given by the 97.2% sulfuric acid solution of I as shown in Fig. 2. It seemed to be plausible that

17) A.M. Smoczkiewicz and R.M. Zalewski, *Steroids*, **12**, 391 (1968).
18) 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.
19) J. Torreilles and A.C. de Paulet, *Bull. Soc. Chim. Fr.*, **1968**, 4886.
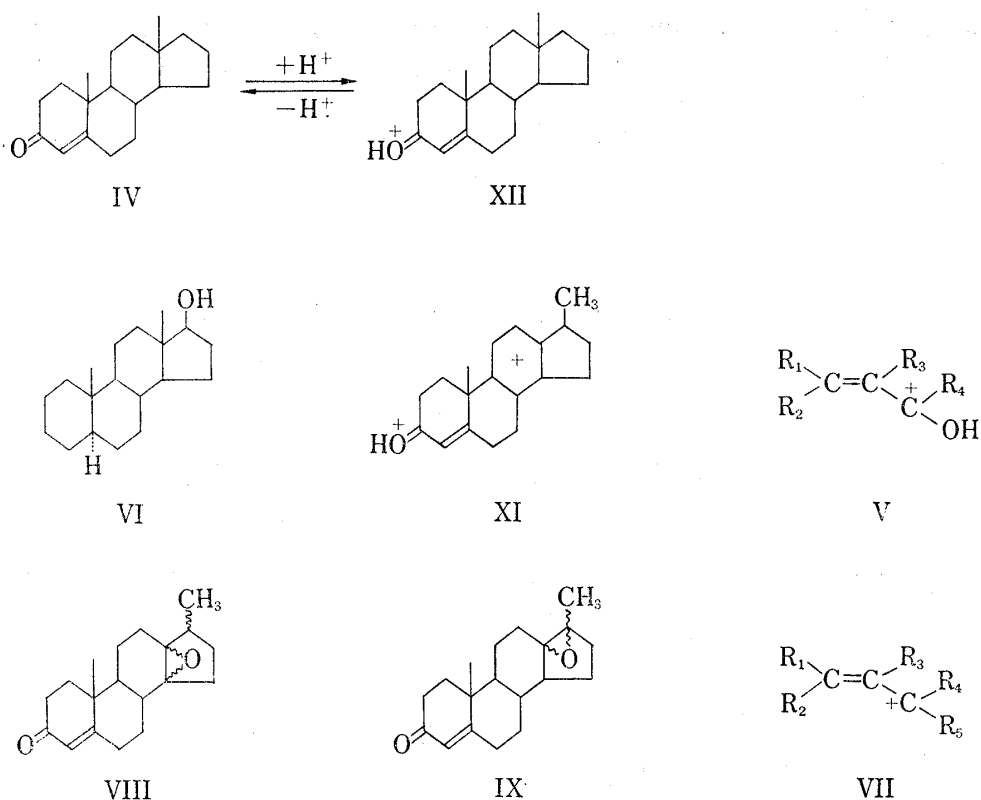
Chart 2

the chromophore, which principally contributes to the absorption at 300 nm besides the hydroxyalkenyl cation (V) moiety, has an alkenyl cation (VII) system, and that the dication (X; designated as X-300) is formed in the reaction of I or the olefin (II+III) with concentrated sulfuric acid.

In addition to these optical data, nuclear magnetic resonance (NMR) spectrum of the 97.2% sulfuric acid solution of I was identical with that of the epoxide (VIII+IX) as shwon in Fig. 3a. The spectrum of the cation (XII) prepared from IV with 97.2% sulfuric acid and those of I and IV in deuteriochloroform are shown in Fig. 3b, 3c, and 3d, respectively. The signals of C(13)–CH$_3$, C(10)–CH$_3$, and C(4)–H of XII appeared at 0.98, 1.51, and 6.85 ppm, respectively. The downfield shifts of C(4)–H and C(10)–CH$_3$ signals of XII relative to those of IV have been proved to result from the inductive deshielding effect of the hydroxyalkenyl cation (V) moiety.[17] On the other hand, lack of the signal due to C(13)–CH$_3$, slight downfield shifts of signals due to C(10)–CH$_3$ as well as C(4)–H, and increase in the relative area of signals in the about 3.0 and 3.5 ppm regions were observed in the NMR spectrum of I in 97.2% sulfuric acid (Fig. 3a), compared with that of XII. Deno et al. observed the NMR spectra of several cyclopentenyl cations.[20] Of these cations, 1-isopropyl-3-methylcyclopentenyl cation (XIII) is of interest in view of its structural similarity to the alkenyl cation moiety of the X-300 (X). The cation is stable in concentrated sulfuric acid and its methyl protons and methylene as well as methine protons situated at α-positions to the alkenyl cation system appeared at about 3.0 and 3.5 ppm regions, respectively. These values were in good agreement with those given in Fig. 3a. It seemed to be reasonable that the signals of C(17)–CH$_3$ and those of C(12)–H$_2$, C(15)–H$_2$, C(16)–H$_2$, and C(8)–H situated at α-positions to the alkenyl cation system of the X-300 (X) is anticipated to appear in the about 3.0 and 3.5 ppm regions, respectively. Thus, the NMR spectrum of I in 97.2% sulfuric

20) N.C. Deno, H.G. Richey, N. Friedman, J.D. Hodge, J.J. Houser, and C.V. Pittman, J. Am. Chem. Soc., 85, 2991 (1963).

acid also supported that X is the carbocation. Jones *et al.* obtained a similar NMR spectrum due to I under similar condition and speculated that I in concentrated sulfuric acid is converted into the carbonium ion (XI) delocalizing the positive charge over several adjacent tertiary carbon atoms by protonation, dehydration, and angular methyl migration but not by oxidation.[21] The interpretation by Jones seems, therefore, to be not satisfactory to our experimental results.
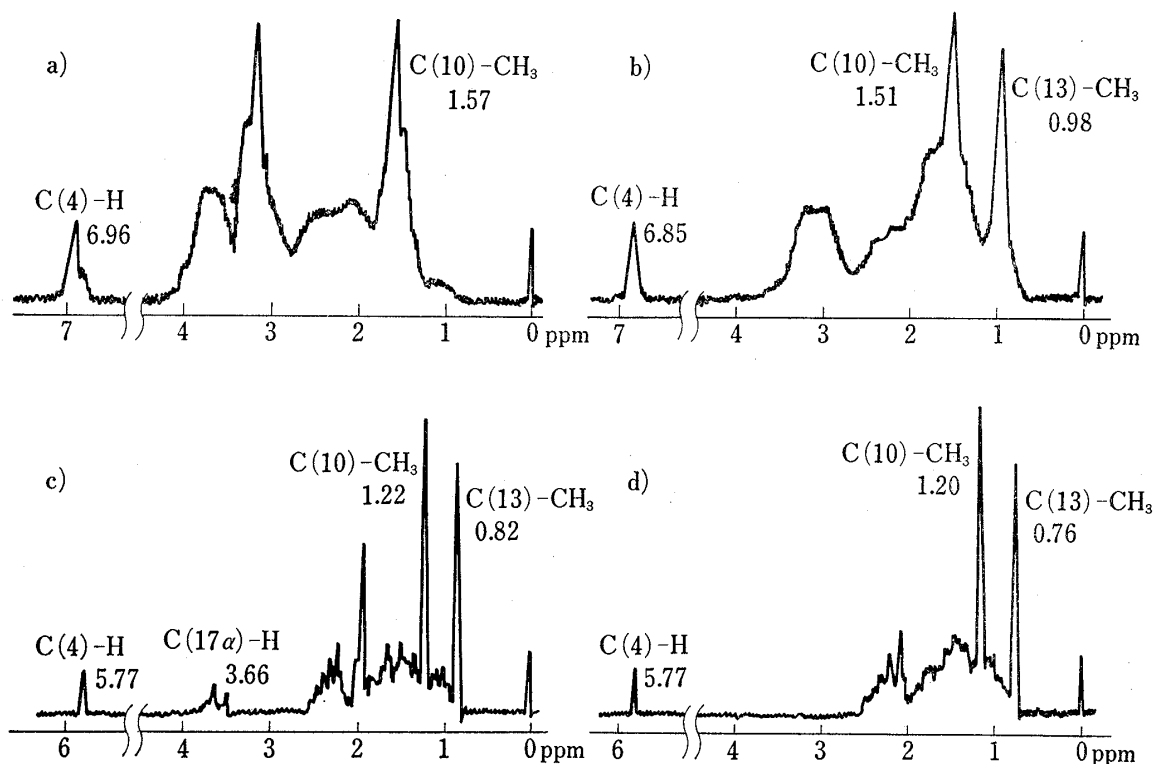

Fig. 3.   NMR spectra of I and IV in 97.2% H_2SO_4 and Those in $CDCl_3$

steroid: 50 mg, solvent: 0.5 ml, 60 Mc, 35°.
ppm from external capillary tetramethylsilane
a) I or VIII+IV in 97.2% H_2SO_4,   b) IV in 97.2% H_2SO_4,
c) I in $CDCl_3$,   d) IV in $CDCl_3$.

In conclusion, the results from these absorption and NMR spectroscopic studies suggest that the primary process of the reaction of testosterone (I) with sulfuric acid proceeds as shown in Chart 2.   Namely, protonation at the C(3)-carbonyl and C(17)-hydroxyl functions takes place and then dehydration at C(17) with subsequent methyl migration from C(13) to C(17) occurs to form the cation (XIV).   Since most alkyl cations are unstable in concentrated sulfuric acid, the cation (XIV) is in turn converted into the stable alkenyl one, the X-300 (X), through oxidation of its conjugate bases (II+III) by sulfuric acid.

Correlation between the chromophoric species X-300 and X-484 will be discussed in a subsequent paper.

Experimental

General Methods——Absorption spectra were measured by Hitachi Model EPS-3T recording spectrometer.   NMR spectra were recorded on Hitachi Model R-20-B at 60 MHz using tetramethylsilane as an internal standard.   Mass spectral measurement was run on Hitachi Model RMU-6R spectrometer.   For preparative thin-layer chromatography (TLC), silica gel (Wakogel B-5-F) was used as an adsorbent.   Abbreviation used s=singlet, d=doublet.

21) H.A. Jones, *J. Chem. Soc.* (*B*), **1971**, 99; H.A. Jones, Ph.D. Thesis, University of Western Australia, 1968.

Materials——Androst-4-en-3-one (IV)[22] and 5α-androstan-17β-ol (VI)[23] were prepared by the methods reported.

A Mixture of 17-Methyl-18-norandrosta-4,13-dien-3-one (II) and 17-Methyl-18-norandrosta-4,13(17)-dien-3-one (III)——The preparative methods reported by Knox *et al.*[14] and Sondheimer *et al.*[15] were simplified for this study as follows: A mixture of testosterone methane sulfonate (1.5 g), acetic acid (30 ml), and potassium acetate was refluxed for 24 hr under nitrogen atmosphere. The reaction mixture was then diluted with water and extracted with ether. The organic layer was washed with 10% NaOH and water successively, dried over Na_2SO_4, and evaporated *in vacuo*. The oily residue obtained was submitted to column chromatography on alumina. Evaporation *in vacuo* of the solvent from the fraction eluted by hexane–benzene (7: 3) gave a mixture of II and III as colorless oil (581 mg). *Anal.* Calcd. for $C_{19}H_{26}O$: C, 84.39; H, 9.69. Found: C, 84.28; H, 9.66. MS *m/e*: 270 (M+). UV λ^{EtOH}_{max} nm (ε): 242 (16000). IR ν^{Nujol}_{max} cm⁻¹: 1675 (C=O). NMR (10% solution in $CDCl_3$) δ: 5.75 (1H, s, C(4)–H), 1.62 (3H, s, C(17)–CH_3 of III), 1.16 (3H, s, C(10)–CH_3 of II), 1.12 (3H, s, C(10)–CH_3 of III), 0.96 (3H, d, J=6 Hz, C(17)–CH_3 of II). From the relative area of C(17)–CH_3 signals of II and III, the oil was proved to be a 1:1 mixture of these olefins.

A Mixture of 13ξ,14ξ-Epoxy-17-methyl-18-norandrost-4-en-3-one (VIII) and 13ξ,17ξ-Epoxy-17-methyl-18-norandrost-4-en-3-one (IX)——A mixture of the dienes (II+III, 500 mg), *m*-chloroperbenzoic acid (320 mg), 0.5 M $NaHCO_3$ (10 ml), and CH_2Cl_2 (10 ml) was stirred for 4 hr at room temperature under nitrogen atmosphere. The reaction mixture was then diluted with 5% Na_2SO_3 and extracted with $CHCl_3$. The organic layer was washed with 5% Na_2SO_3, 5% $NaHCO_3$, and water successively and dried over anhydrous Na_2SO_4. After worked up as usual, the oily residue obtained was submitted to preparative TLC using benzene–acetone (4: 1) as developing solvent. The adsorbent corresponding to the spot of *Rf* 0.55 was eluted with $CHCl_3$ and the eluate was dissolved into MeOH. The solution was cooled at 0° to give a 1:1 mixture of VIII and IX as a powder. *Anal.* Calcd. for $C_{19}H_{26}O$: C, 79.68; H, 9.15. Found: C, 79.52; H, 9.08. MS *m/e*: 286 (M+). UV λ^{EtOH}_{max} nm (ε): 242 (16000). NMR (10% solution in $CDCl_3$) δ: 5.78 (1H, s, C(4)–H), 1.38 (3H, s, C(17)–CH_3 of IX), 1.21 (3H, s, C(10)–CH_3 of VIII or IX), 1.13 (3H, s, C(10)–CH_3 of IX or VIII), 1.11 (3H, d, J=6 Hz, C(17)–CH_3 of VIII). From the relative area of C(17)–CH_3 signals of VIII and IX, the product was proved to be a 1:1 mixture of these epoxides.

Absorption Spectrum in Sulfuric Acid——To a dried sample (50—100 μg) was added sulfuric acid (5 ml) at room temperature. The mixture was shaken vigorously to make a homogeneous solution and measured on a spectrometer at 25°.

NMR Spectrum of Steroidal Carbocation in Concentrated Sulfuric Acid——To a sample (50 mg) was added 97.2% H_2SO_4 (0.5 ml) and the mixture was shaken vigorously to make a homogeneous solution. NMR spectrum was recorded with external capillary tetramethylsilane as a reference at 35°. No spectral change was detected in each sample examined, when it was diluted further with 97.2% H_2SO_4 after NMR measurement. In the case of IV, the starting material was recovered quantitatively after the measurement, which was confirmed by TLC and NMR spectrum.

22) R.H. Shapiro and C. Djerassi, *J. Am. Chem. Soc.*, **86**, 2825 (1964).
23) L. Caglioti, *Org. Syn.*, **52**, 122 (1972).