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Further Studies on the Mechanism of the Color and Fluorescence Reaction of Testosterone with Sulfuric Acid[1)]

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The chemical structure of the chromo- and fluorophoric species χ-600 ($\lambda_{max}=600$ nm, $\lambda_{em}=615$ nm), which is formed in the reaction of testosterone (I) with sulfuric acid, was elucidated. The chemical species χ-600 was shown to be produced from the intermediary χ-300 ($\lambda_{max}=300$ nm) through oxidation of its conjugate base (IV). 17-Methyl-18-norandrosta-4,6,8(14),13(17)-tetraen-3-one (X), the conjugate base of χ-600, was isolated from the reaction of 17β-hydroxyandrosta-4,6-dien-3-one (VIII) with a mixture of selenic acid, sulfuric acid, and ethanol. The ^{1}H-nuclear magnetic resonance spectrum of X in 53% deuterio sulfuric acid indicated that χ-600 is a hydroxyalkatetraenyl cation (VII). On the basis of the results described above, a mechanism is proposed for the color and fluorescence reaction.

Keywords——^{1}H-NMR; hydroxyalkatrienyl cation; hydroxyalkatetraenyl cation; mechanism of color and fluorescence reaction; 17-methyl-18-norandrosta-4,6,8(14)-trien-3-one; 17-methyl-18-norandrosta-4,6,8(14),13(17)-tetraen-3-one; oxidation with selenic acid; steroidal carbocation; sulfuric acid; testosterone

Although the color and fluorescence reactions of steroidal 4-en-3-ones such as testosterone (I) and corticoids with sulfuric acid have been widely used for qualitative and quantitative determinations, the mechanism of these reactions remains mostly unknown.[2)] In our preceding papers of this series, the dication (II, $\lambda_{max}=300$ nm, abbreviated as χ-300) was demonstrated to be produced at the early stage[1b,3)] and then transformed into two main chromophoric species[4)] in the reaction of I with sulfuric acid. One of these species was shown to be a hydroxyalkatrienyl cation (IIIa, $\lambda_{max}=484$ nm, $\lambda_{em}=530$ nm, abbreviated as χ-484) which is produced by acid-catalyzed isomerization of χ-300.[4,5)] In this report, the chemical structure and formation mechanism of another chromophoric species ($\lambda_{max}=600$ nm, $\lambda_{em}=615$ nm, abbreviated as χ-600) are discussed.

Results and Discussion

When a solution of χ-300 obtained by dissolving I in 97% sulfuric acid was diluted with water or ethanol, two chromophoric species, χ-484 (IIIa) and χ-600, were produced as shown in Fig. 1. Upon dilution of the same acid solution with water or ethanol containing selenic acid, the formation of χ-600 predominated over that of IIIa. Since an oxidative process participates in the conversion of χ-300 (II) to χ-600, the solution of II was diluted with ten volumes of 53% sulfuric acid containing various amounts of selenic acid and the time course of apparent molar absorptivity of the resulting solutions at 600 nm was measured. As shown in Fig. 2, the conversion of II was accelerated as the concentration of selenic acid was increased. The accelerating effect of selenic acid was, on the other hand, found to be larger in systems of lower acid strength, as shown in Fig. 3. These results indicated that the conversion of χ-300 (II) to χ-600 is an oxidative process *via* the conjugate base (IV) of II.

Dissolution of an equimolar mixture of 13ξ,17ξ-epoxy-17ξ-methyl-18-norandrost-4-en-3-one (V) and 13ξ,14ξ-epoxy-17ξ-methyl-18-norandrost-4-en-3-one (VIa) in 97% sulfuric acid gave χ-300 (II), which was converted to χ-484 (IIIa) and χ-600 by dilution with water as previously reported.[4] The mixture of the epoxides (V and VIa) also produced IIIa as well as χ-600 on being dissolved in 50% sulfuric acid, and the latter product was predominant when selenic acid was added to this system. Though 17,17-dimethyl-13ξ,14ξ-epoxy-18-norandrost-4-en-3-one (VIb) also gave χ-484 (IIIb) on being dissolved in the same acid, no formation of χ-600 was observed in spite of the presence of selenic acid (Fig. 4). Tertiary nature of C(17) seemed, therefore, to be necessary for formation of χ-600. Taking into consideration that the conversion of χ-300 to χ-600 is an oxidative process, the latter species was assumed to be a hydroxyalkatetraenyl cation (VII).

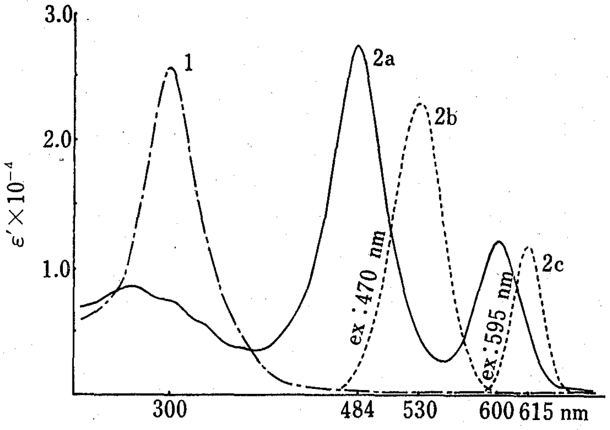

Fig. 1. Conversion of χ-300 (II) to χ-484 (IIIa) and χ-600 (VII)

1: I (50 μg) was dissolved in 5 ml of 97% H_2SO_4. Absorption spectrum.

2: the same solution of I (2 ml) was poured into ethanol (3 ml).
2a) absorption spectrum.
2b and 2c) fluorescence spectra with excitation wavelengths of 470 and 595 nm, respectively.

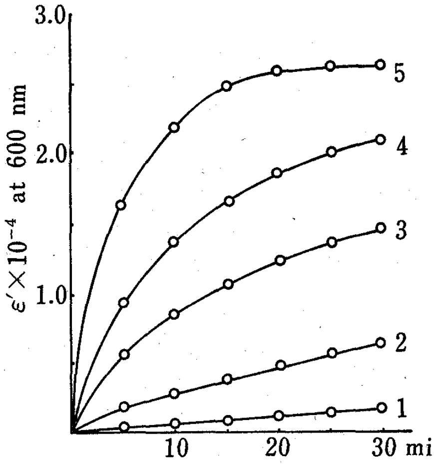

Fig. 2. Effect of Selenic Acid on the Formation of χ-600 (VII)

A 97% H_2SO_4 solution (0.5 ml) of VIII (106 μg/ml) was poured into 5 ml of 53% H_2SO_4 containing H_2SeO_4. The final concentrations of H_2SeO_4 were: 1 (0%), 2 (0.002%), 3 (0.003%), 4 (0.005%), and 5 (0.011%).

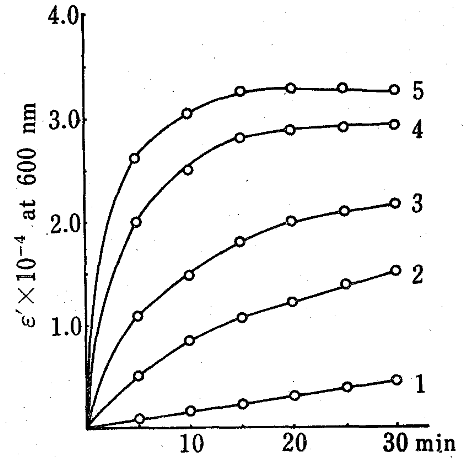

Fig. 3. Effect of Selenic Acid on the Formation of χ-600 (VII) in Sulfuric Acid of Various Concentrations

A 97% H_2SO_4 solution (0.5 ml) of VIII (106 μg/ml) was poured into 5 ml of H_2SO_4 of various concentrations containing H_2SeO_4. The final concentration of H_2SeO_4 was 0.008%. The final concentrations of H_2SO_4 were: 1 (60%), 2 (55%), 3 (50%), 4 (45%), 5 (40%).

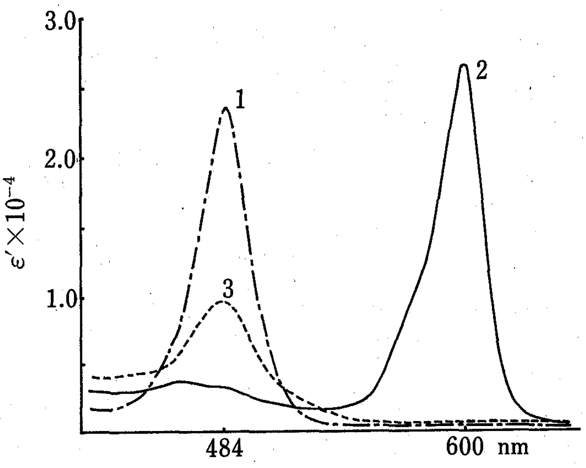

Fig. 4. Absorption Spectra of the Epoxides (V, VIa, VIb) in 50% Sulfuric Acid containing Selenic Acid

1: a mixture of V and VIa (66 μg) was dissolved in 5 ml of 50% H_2SO_4.

2: the same acid solution of V and VIa containing 0.004% H_2SeO_4.

3: the same acid solution of VIb containing 0.004% H_2SeO_4.

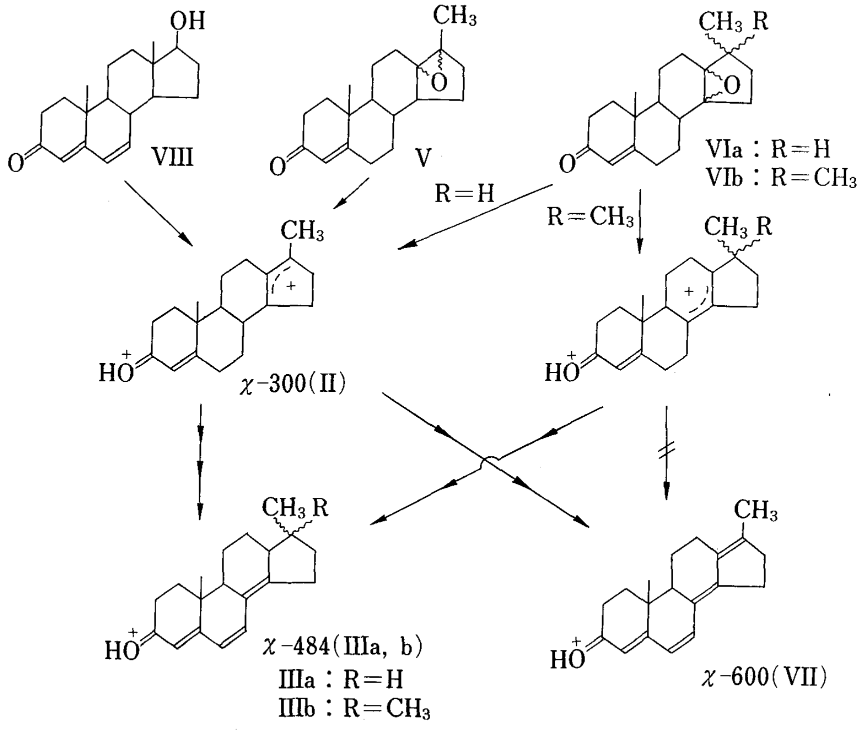

Chart 1

In order to obtain further evidence for the structure of χ-600, appropriate reaction conditions were investigated spectroscopically for the isolation of the conjugated base of χ-600. Dissolution of 17β-hydroxyandrosta-4,6-dien-3-one (VIII) in 97% sulfuric acid immediately gave χ-300 (II) as previously reported,[1b] and this was converted mainly to χ-484 (IIIa) by dilution with 1.5 volumes of ethanol. On the other hand, χ-600 was shown to be predominantly produced upon dilution of the same solution with the same volume of ethanol containing selenic acid (Fig. 5). On the basis of these results, the solution of IIIa was prepared on a preparative scale and poured into excess ice-water to yield an isomeric mixture of 17-methyl-18-norandrosta-4,6,8(14)-trien-3-one (IXa$_1$ and IXa$_2$), the conjugated base of IIIa, in 29% yield. Crystallization of the product from methanol gave 17β-methyl-18-norandrosta-4,6,8(14)-trien-3-one (IXa$_1$) as yellow needles, mp 157—162°C, *m/e*: 268 (M$^+$), $\lambda_{\max}^{\mathrm{EtOH}}=351$ nm ($\varepsilon=25500$), $\nu_{\mathrm{c=o}}$: 1660 cm^{-1}.

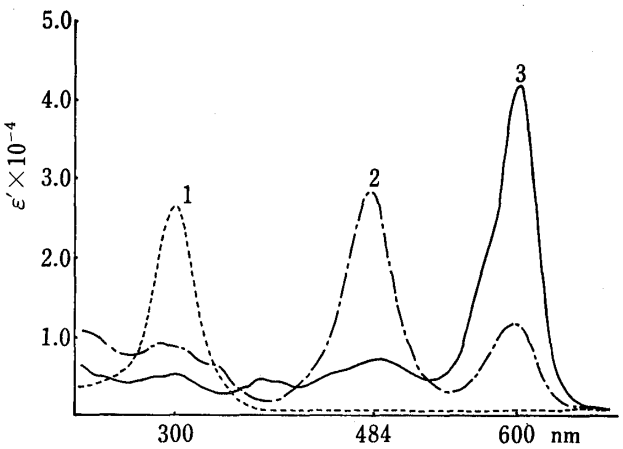

Fig. 5. Formation of χ-484 (IIIa) and χ-600 (VII) from χ-300 (II) in Sulfuric Acid–Ethanol Mixture in the Absence or Presence of Selenic Acid

1: VIII (56 μg) was dissolved in 5 ml of 97% H_2SO_4.
2: The solution of 1 (2 ml) was poured into ethanol (3 ml).
3: The solution of 1 (2 ml) was poured into ethanol (3 ml) containing H_2SeO_4 (final concentration: 0.006%).

On the other hand, a brown oil, *m/e*: 266 (M$^+$), $\lambda_{\max}^{\mathrm{EtOH}}=410$ nm ($\varepsilon=23600$), $\nu_{\mathrm{c=o}}$: 1650 cm^{-1}, was obtained in 19% yield when 97% sulfuric acid solution of VIII was diluted with 1.5 volumes of ethanol containing 2% selenic acid followed by work-up as described above. An additional conjugated double bond was, thus, likely to be introduced into the conjugate base

(IXa_1) of χ-484 (IIIa). In the nuclear magnetic resonance (NMR) spectrum of this oil, the only vinylic proton signals were those due to C(4)-, C(6)-, and C(7)-H, but a signal due to C(17)-CH_3 as a vinylic methyl group appeared at 1.84 ppm. The ^{13}C-NMR spectrum gave nine signals due to the sp^2 carbons of C(3), C(4), C(5), C(6), C(7), C(8), C(13), C(14), and C(17). These spectral data revealed that the oil is 17-methyl-18-norandrosta-4,6,8(14),13(17)-tetraen-3-one (X), the conjugate base of χ-600 (VII). Dissolution of the trienone (IXa_1) and tetraenone (X) thus obtained in a mixture (2: 3, v/v) of sulfuric acid and ethanol showed, as expected, absorption maxima at 484 (ε=39200) and 600 nm (ε=45800) with fluorescence maxima at 530 and 615 nm, respectively (Fig. 6).

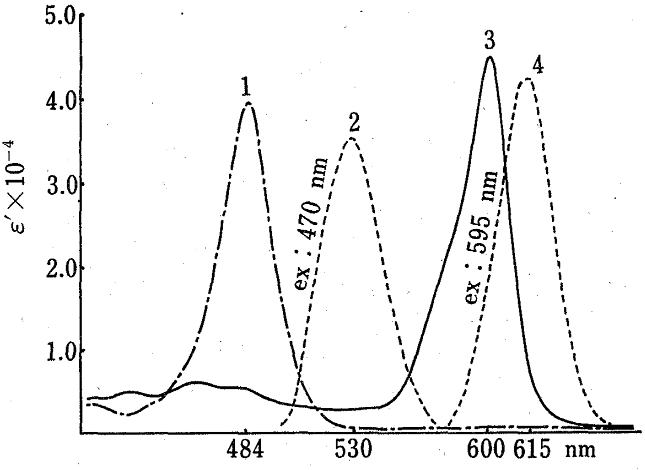

Fig. 6. Absorption and Fluorescence Spectra of χ-484 (IIIa) and χ-600 (VII) in Sulfuric Acid–Ethanol Mixture (2: 3, v/v)

IXa_1 or X (40 μg) was dissolved in 5 ml of H_2SO_4–ethanol (2: 3, v/v) mixture. 1: absorption spectrum of χ-484 (IIIa). 2: fluorescence spectrum of χ-484 with excitation wavelength at 470 nm. 3: absorption spectrum of χ-600 (VII). 4: fluorescence spectrum of χ-600 with excitation wavelength at 595 nm.

Direct evidence for the structure of χ-600 (VII) was, furthermore, obtained by ^1H-NMR spectroscopic studies as described below. In the spectrum obtained four minutes after dissolving the tetraenone (X) in 53% deuterio sulfuric acid,[6] the signals due to C(4)-, C(6)-, C(7)-H, and C(17)-CH_3 were markedly shifted to lower field, in contrast to those of X in $CDCl_3$. Compared with the chemical shifts (Table I) of the corresponding signals due to χ-484 (IIIa), upfield shifts (0.39, 0.25, and 0.14 ppm) of the signals due to vinyl protons at C(4), C(6), and C(7) were observed with a downfield shift (0.91 ppm) of that due to C(17)-CH_3. These data fully support the view that χ-600 is a hydroxyalkatetraenyl cation (VII). Hydrogen-deuterium exchange at C(4) and C(6) of χ-600 occurred so easily that the resultant singlet signal due to

TABLE I. ^1H-NMR Spectral Data for χ-484 (IIIa), χ-600 (VII) and Their Conjugate Bases (IXa_1, X)

	C(4)-H	C(6)-H	C(7)-H	C(17)-CH_3
IIIa[a]	6.59	6.68, d, (J=8 Hz)	7.74, d, (J=8 Hz)	1.20
VII[b]	6.20	6.43, d, (J=8 Hz)	7.60, d, (J=8 Hz)	2.11
IXa_1[c]	5.73	6.02, d, (J=10 Hz)	6.61, d, (J=10 Hz)	1.07, d, (J=6 Hz)
X[c]	5.73	6.02, d, (J=10 Hz)	6.63, d, (J=10 Hz)	1.84

a) In 60% D_2SO_4, ppm from external capillary tetramethylsilane.
b) In 53% D_2SO_4.
c) In $CDCl_3$, ppm from internal tetramethylsilane.

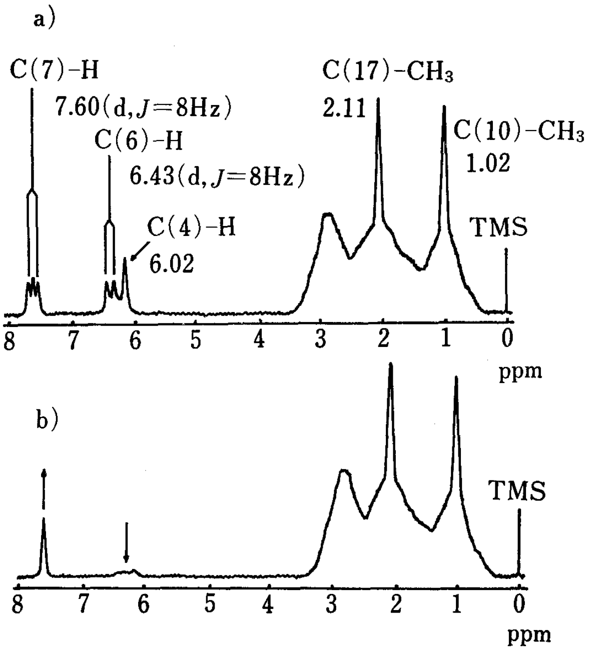

Fig. 7. Changes in the ^{1}H-NMR Spectra of χ-600 (VII) in 53% Deuterio Sulfuric Acid with Time

X (30 mg) was dissolved in 0.5 ml of 53% D_2SO_4; spectra were obtained at 60 MHz, 35°C, ppm from external capillary tetramethylsilane.

a) 4 min and b) 20 min after dissolution.

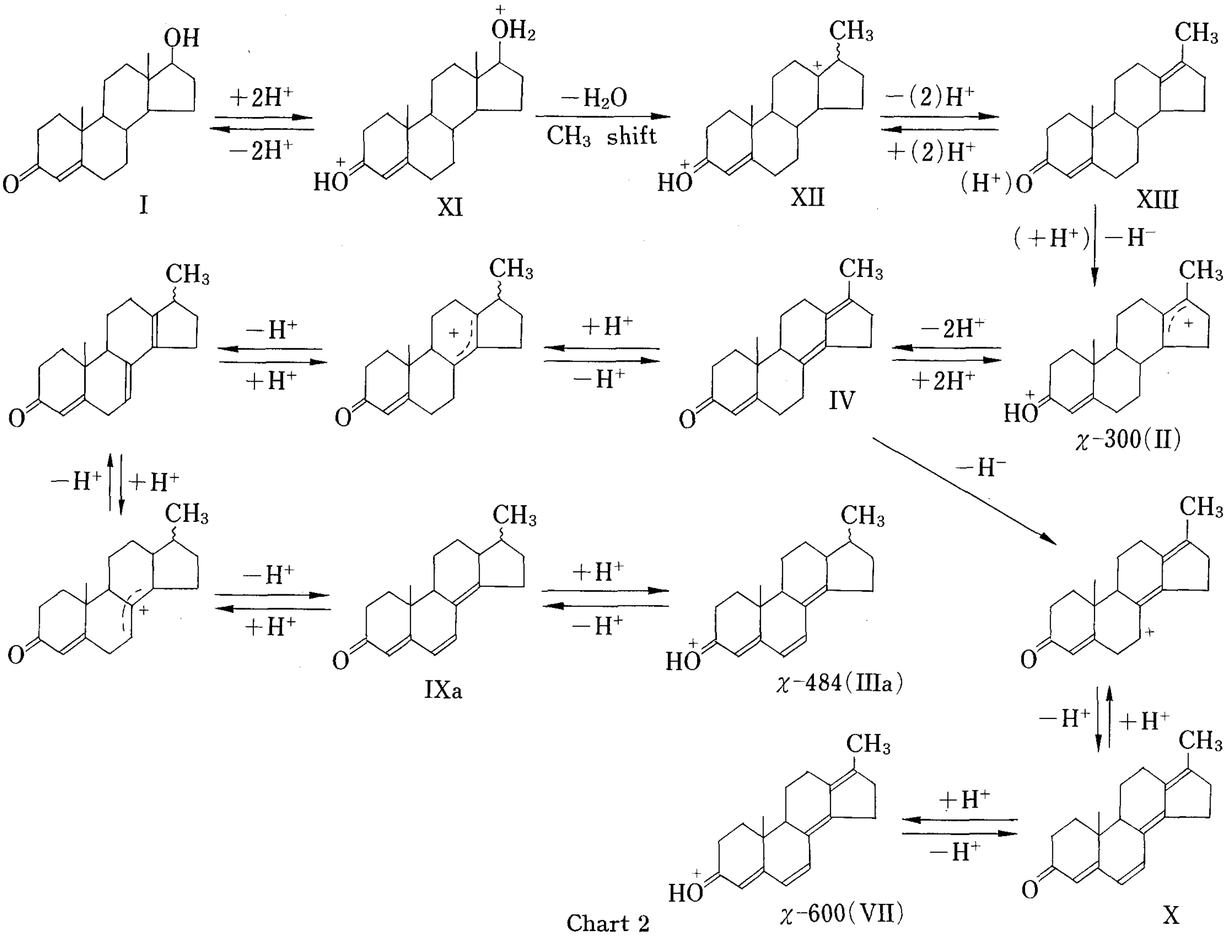

Chart 2

C(7)-H was observed even four minutes after mixing (Fig. 7a). The signals due to C(4)- and C(6)-H then decreased with increase in the singlet, in place of the doublet, signal due to C(7)-H and finally disappeared completely within twenty minutes after mixing (Fig. 7b). From the ^{1}H-NMR chemical shifts and the structure of canonical forms of χ-600, the positive charge density at C(7) may reasonably be assumed to be higher than those at C(4) and C(6). It is, therefore, reasonable that hydrogens at C(4) and C(6) are readily exchangeable by deuterium.

It may be concluded on the basis of the facts described above that the color and fluorescence reactions of testosterone (I) with sulfuric acid proceed as shown in Chart 2. Both oxygen atoms of the C(3)-carbonyl and C(17)-hydroxyl group are protonated to form XI (λ_{max}=292 nm, ε=16700)[1b] which is then converted into XII by dehydration and methyl migration from C(13) to C(17). Because of the instability even in concentrated sulfuric acid, the alkyl cation (XII) transforms into χ-300 (II), which has a more stable cyclopentenyl cation moiety, through oxidation of the conjugate base (XIII).[3,7] The chemical species II is the intermediate which can initially be observed spectrophotometrically in the reaction of I with sulfuric acid. Since the estimated value of pK_a of II is about -3,[4] it is stable in sulfuric acid of high concentration. Decrease in the concentration of acid, on the other hand, shifts the equilibrium between χ-300 and its conjugate base (IV) to the side of IV. The acid-catalyzed isomerization[4] and oxidation of IV thus occur competitively, forming two chromo- and fluorophoric species, χ-484 and χ-600 (VII), respectively.

Experimental

General Methods——All melting points were taken on a micro hot-stage apparatus and are uncorrected. Silica gel (Wakogel B5F) was used as an adsorbent for the preparative thin-layer chromatography (TLC). Absorption and fluorescnce spectra were measured with a Shimadzu UV-220 recording spectromter and a Hitachi MPF-3 fluorescence spectrometer, respectively. Mass (MS) spectral measurements were run on a JEOL JMS-D-300 spectrometer. Infrared (IR) spectra were taken on a JASCO A-102 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Hitachi R-20-B spectrometer at 60 MHz and a JEOL JNM FX 100 FT spectrometer at 100 MHz, respectively, using tetramethylsilane as an internal standard. Abbreviations used: s=singlet, d=doublet. Absorption and ^{1}H-NMR spectra of steroids in sulfuric acid were obtained according to the reported method.[1b]

Materials——Testosterone (I) was purchased commercially and used after recrystallization. An equimolar mixture of 13ξ,17ξ-epoxy-17ξ-methyl-18-norandrost-4-en-3-one (V) and 13ξ,14ξ-epoxy-17ξ-methyl-18-norandrost-4-en-3-one (VIa) was prepared as reported in the previous paper.[3] 17,17-Dimethyl-13ξ,14ξ-epoxy-18-norandrost-4-en-3-one (VIb)[8] and 17β-hydroxyandrosta-4,6-dien-3-one (VIII)[9] were prepared by the cited methods.

Isolation of the Conjugate Bases (IXa and X) of χ-484 (IIIa) and χ-600 (VII)——17-Methyl-18-norandrosta-4,6,8(14)-trien-3-one (IXa): A mixture of sulfuric acid (97%, 10 ml) and VIII (300 mg) was shaken vigorously to give a homogenous solution and then allowed to stand at room temperature for 20 min. The reaction mixture was diluted with EtOH (15 ml) under ice-cooling. The resulting red-brown solution was then gradually poured into ice-water under vigorous stirring and extracted with ether (200 ml × 3). The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue thus obtained was subjected to preparative TLC and developed with benzene–acetone (4: 1). Elution of the adsorbent corresponding to the spot of *Rf* 0.48 with ether gave 83 mg of IXa$_1$, IXa$_2$ as a yellow oil, which was shown to be a mixture of two isomers by the presence of two signals due to C(17)–CH_3 at 1.07 and 0.69 ppm (3: 1) in the ^{1}H-NMR spectrum. Crystallization of the oil from MeOH gave one of the isomers, 17β-methyl-18-norandrosta-4,6,8(14)-trien-3-one (IXa$_1$),[2c] as yellow needles, mp 157—162°C. *Anal.* Calcd for $C_{19}H_{24}O$: C, 85.02; H, 9.01. Found: C, 84.81; H, 9.07. λ_{max}^{EtOH} nm (ε): 351 (25500). MS *m/e*: 268 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1660 (C=O). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 6.61 (1H, d, J=10 Hz, C(7)–H), 6.02 (1H, d, J=10 Hz, C(6)-H), 5.73 (1H, s, C(4)-H), 1.07 (3H, d, J=6 Hz, C(17β)–CH_3), 0.99 (3H, s, C(10)–CH_3). ^{13}C-NMR (10% solution in $CDCl_3$) δ: 199.4(3), 122.9(4), 164.5(5), 124.2(6), 133.8(7), 125.3(8), 151.4(14).

17-Methyl-18-norandrosta-4,6,8(14),13(17)-tetraen-3-one (X): A solution of VIII (300 mg) in sulfuric acid (97%, 10 ml) was prepared as described above and diluted with EtOH (15 ml) containing 2% selenic acid to give a deep purple solution. The solution thus obtained was worked up as usual and subjected to preparative TLC in a manner similar to that described above. Elution of the adsorbent corresponding to the spot of *Rf* 0.44 with ether gave 54 mg of 17-methyl-18-norandrosta-4,6,8(14),13(17)-tetraen-3-one (X) as a red-brown oil. $\lambda_{\max}^{\mathrm{EtOH}}$nm (ε): 410 (23600). MS *m/e*: 266 (M^+). IR $\nu_{\max}^{\mathrm{neat}}$ cm^{-1}: 1650 (C=O). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 6.63 (1H, d, *J*=10 Hz, C(7)–H), 6.02 (1H, d, *J*=10 Hz, C(6)–H), 5.73 (1H, s, C(4)–H), 1.84 (3H, s, C(17)–CH_3), 1.03 (3H, s, C(10)–CH_3). ^{13}C-NMR (10% solution in $CDCl_3$) δ: 199.1(3), 164.5(5), 152.3(14), 144.6(17), 135.9(13), 134.0(7), 122.7(6), 122.5(8), 122.1(4).

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References and Notes

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