[Chem. Pharm. Bull. 33(8)3317—3323(1985)]

On the Mechanism of Color and Fluorescence Reaction of 17α-Hydroxyprogesterone with Sulfuric Acid[1)]

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(Received December 17, 1984)

The mechanism of the color and fluorescence reaction of 17α-hydroxyprogesterone (**1**) with sulfuric acid was elucidated. When a 97% sulfuric acid solution of **1** was heated at 60 °C for 45 min and then diluted two-fold with ethanol, a chromo- and fluorophoric species ($\lambda_{max}=592$ nm, $\lambda_{em}=614$ nm, abbreviated as χ-592) was produced. From this reaction mixture, 17ξ,17a-dimethyl-18-nor-D-homoandrosta-4,6,8(14),13(17a)-tetraen-3-one (**4a**) was isolated in 13% yield. Dissolution of the tetraenone (**4a**) in a 1 : 2 mixture of sulfuric acid and ethanol immediately gave an absorption maximum at 592 nm ($\varepsilon=35200$) with fluorescence at 614 nm. The proton nuclear magnetic resonance spectrum of **4a** in a 1 : 2 mixture of D_2SO_4 and CD_3OD indicated that the chromo- and fluorophoric χ-592 is a hydroxyalkatetraenyl cation (**4b**), the protonated form of **4a**. χ-592 (**4b**) was shown to be formed from **1** *via* an intermediary species ($\lambda_{max}=427$ nm, $\lambda_{em}=472$ nm). The structure of the intermediary species was also assumed to be a steroidal dication (**9**) having both a hydroxyalkenyl cation in ring A and an alkadienyl cation across rings C and D.

Keywords——17α-hydroxyprogesterone; color reaction; fluorescence reaction; sulfuric acid; mechanism; steroidal tetraenone; D-homoannulation; steroidal carbocation

A number of steroidal compounds to produce color and fluorescence upon reaction with a strong acid. These reactions have served as methods for qualitative and quantitative determinations of the steroids. However, the mechanisms of these reactions have remained unknown. Recently, we elucidated the mechanisms of the color and fluorescence reactions of steroidal estrogens,[2)] testosterone[3)] and progesterone[4)] with sulfuric acid; in all cases, protonation, dehydration, rearrangement and oxidation occur to give chromo- and fluorophoric steroidal carbocations.

In the preceding paper of this series, we also investigated the behavior of 17α-hydroxyprogesterone (**1**) in sulfuric acid in relation to the mechanism of its color and fluorescence reaction.[1b)] On the basis of product analysis and proton nuclear magnetic resonance (^{1}H-NMR) studies, the reaction was concluded to proceed as shown in Chart 1. D-Homoannulation of **1** rapidly occurs to give 17aβ-hydroxy-17aα-methyl-D-homoandrost-4-ene-3,17-dione (**2a**), which is then dehydrated to 17a-methylene-D-homoandrost-4-ene-3,17-dione (**3a**). The protonated forms (**2b** and **3b**, abbreviated as χ-284) of **2a** and **3a** showed an absorption maximum at 284 nm in sulfuric acid. Both **2a** and **3a** are then converted to an intermediary species ($\lambda_{max}=427$ nm, $\lambda_{em}=472$ nm, abbreviated as χ-427) leading to the chromo- and fluorophore ($\lambda_{max}=592$ nm, $\lambda_{em}=614$ nm, abbreviated as χ-592). In this report, we discuss the chemical structures of χ-427 and χ-592, and the mechanism of their formation.

Results and Discussion

As reported previously,[1b)] dissolution of **1** in 97% sulfuric acid gave χ-427 as well as χ-

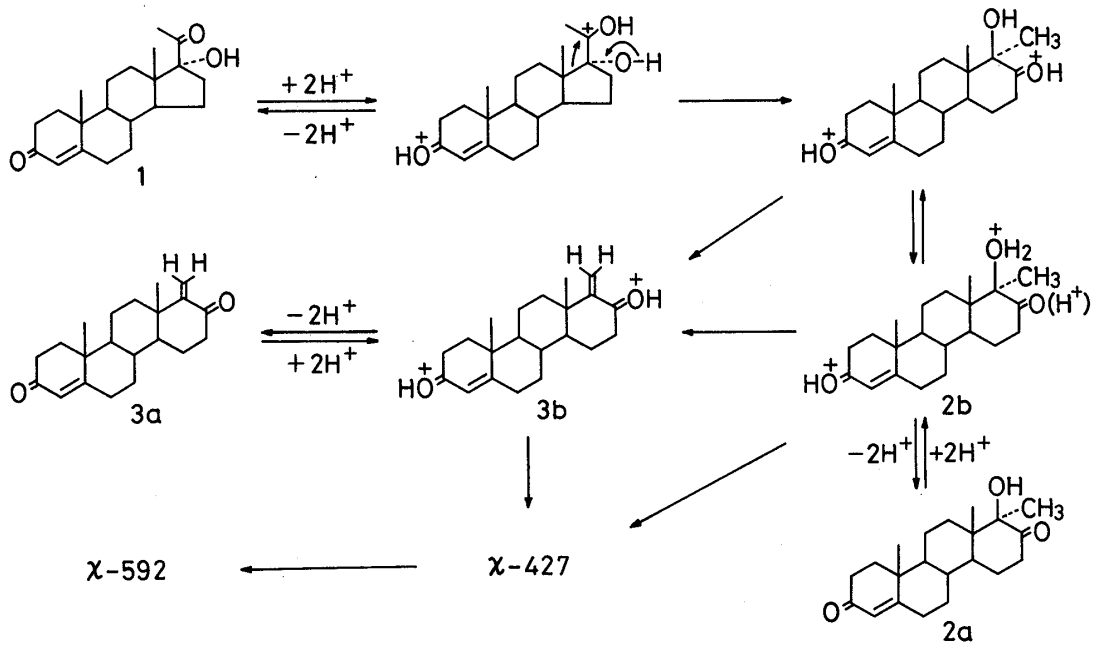

Chart 1. D-Homoannulation of 17α-Hydroxyprogesterone (**1**) with Sulfuric Acid

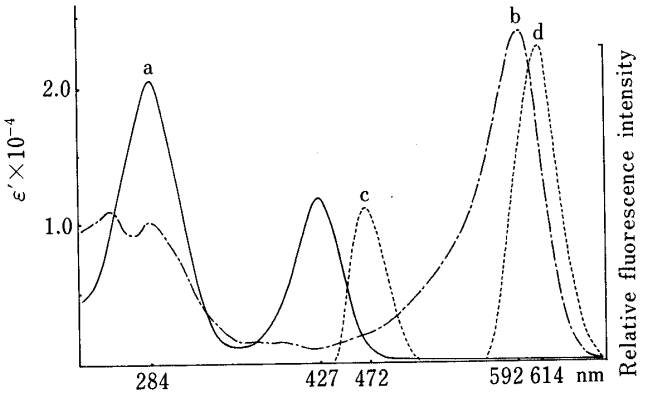

Fig. 1. Absorption and Fluorescence Spectra of 17α-Hydroxyprogesterone (**1**) with Sulfuric Acid and with Sulfuric Acid–Ethanol

a) A mixture of **1** (51 μg) and 97% H_2SO_4 (4 ml) was heated at 60 °C for 45 min. Absorption spectrum. ε′ = apparent molar absorptivity. b) The reaction mixture (a, 2 ml) was poured into ethanol (4 ml) at room temperature. Absorption spectrum. c) Fluorescence spectrum of solution (a) with excitation at 430 nm. d) Fluorescence spectrum of solution (b) with excitation at 589 nm.

284. The formation of χ-427 was accelerated by heating, and the conversion of χ-427 to χ-592 increased with decrease in the acid strength of the medium by diluting it with water or ethanol. Figure 1 shows the absorption and fluorescence spectra of χ-284 and χ-427 obtained by heating a 97% sulfuric acid solution of **1** at 60 °C for 45 min, and of χ-592 obtained by diluting the acid solution two-fold with ethanol. In the same manner, the colored solution of χ-592 was prepared on a preparative scale and poured into excess ice-water. The mixture was then extracted with ethyl acetate and separated by preparative thin-layer chromatography (TLC) to give compound **4a** as an oil in 13% yield. The mass spectrum of **4a** showed a molecular ion peak at *m/e* 294, indicating the loss of two moles of water from **1**. Its infrared (IR) spectrum has signals due to a conjugated carbonyl group and an olefinic bond but no signal due to a hydroxyl group. The visible spectrum of **4a** showed an absorption maximum at 400 nm (ε = 20700) characteristic of a conjugated tetraenone moiety, which is supported by the strong band at 1660 cm^{-1} due to the conjugated carbonyl group. The ^{1}H-NMR spectrum of **4a** showed signals due to the olefinic protons at C(4), C(6) and C(7) at 5.73, 6.08 and 6.88 ppm, respectively. In the spectrum, a doublet signal due to a methyl group and a singlet signal due to a vinyl methyl group were also observed, but the signal of the angular methyl group at C(13) had disappeared. As reported previously,[1b] D-homoannulation of **1** occurred rapidly in

sulfuric acid and χ-592 was also produced from the D-homosteroids, **2a** and **3a**, *via* χ-427. Taking into consideration these facts and the spectral data described above, compound **4a** can be tentatively assigned as 17ξ,17a-dimethyl-18-nor-D-homoandrosta-4,6,8(14),13(17a)-tetraen-3-one, though the configuration of the methyl group at C(17) remains uncertain.

Steroidal tetraenones (**5a** and **6a**) were also isolated previously from the reaction mixtures of testosterone (**7**) and progesterone (**8**) with sulfuric acid.[3d,4] The spectral data for **4a**, **5a** and **6a** are summarized in Table I. Though these data show some differences from each other depending on the structure of ring D, the assigned structure of **4a** is supported by comparison of the data for **4a** with those for **5a** and **6a**.

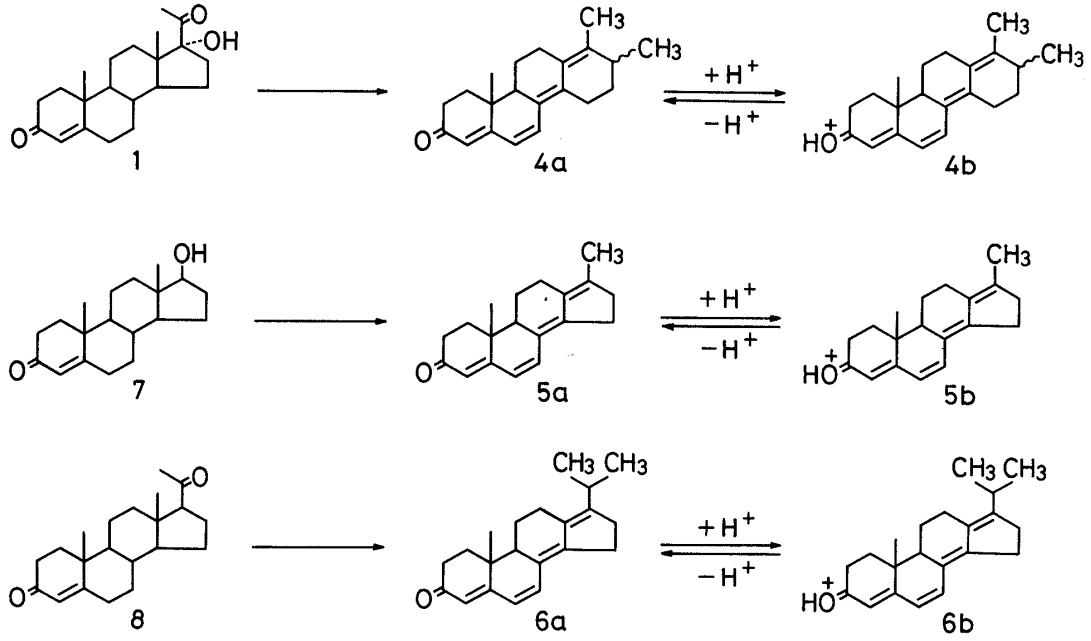

Chart 2. Steroidal Tetraenones and Hydroxyalkatetraenyl Cations

TABLE I. Spectral Data for Steroidal Tetraenones

Tetraenone	λ_{max}^{EtOH}, nm (ε)	$\nu_{C=O}^{neat}$, cm^{-1}	^{1}H-NMR, δ (in $CDCl_3$)					
			C(4)-H	C(6)-H	C(7)-H	C(17)-CH_3	C(17a)-CH_3	C(10)-CH_3
4a	400 (20700)	1660	5.73	6.08	6.88	1.07	1.79	1.00
5a	410 (23600)	1650	5.73	6.02	6.63	1.84		1.03
6a	412 (21000)	1650	5.73	6.01	6.63			1.03

Dissolution of **4a** in a 1:2 mixture of sulfuric acid and ethanol immediately gave an absorption maximum at 592 nm (ε=35200) and fluorescence at 614 nm, as shown in Fig. 2. Therefore, χ-592 is assumed to be the hydroxyalkatetraenyl cation (**4b**) produced by protonation of the carbonyl oxygen at C(3) of **4a**, as in the cases of testosterone[3d] and progesterone.[4] In order to confirm the structure of χ-592, the ^{1}H-NMR spectrum of **4a** in a 1:2 mixture of D_2SO_4 and CD_3OD was measured and is shown in Fig. 3. The spectrum showed a signal-pattern similar to that of **4a** in $CDCl_3$. Compared with the signals of **4a**, all the olefinic proton signals and the vinyl methyl signal were shifted to lower field, while the methyl signals at C(10) and C(17) were shifted to higher field. The extents of the signal shifts from the values of **4a** were in the following order (ppm): C(7)-H (1.08) > C(4)-H (0.59) > C(6)-H (0.45) > C(17a)-CH_3 (0.08) > C(17)-CH_3 (−0.05) > C(10)-CH_3 (−0.11). Consider-

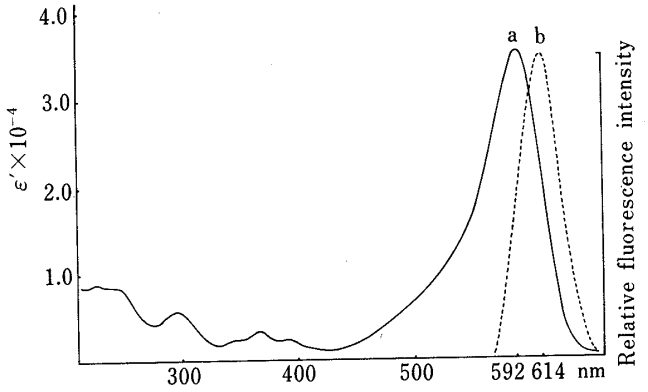

Fig. 2. Absorption and Fluorescence Spectra of χ-592 in a 1:2 Mixture of Sulfuric Acid and Ethanol

4a (22 μg) was dissolved in 4 ml of H_2SO_4–EtOH (1:2, v/v) mixture. a) Absorption spectrum. b) Fluorescence spectrum with excitation at 589 nm.

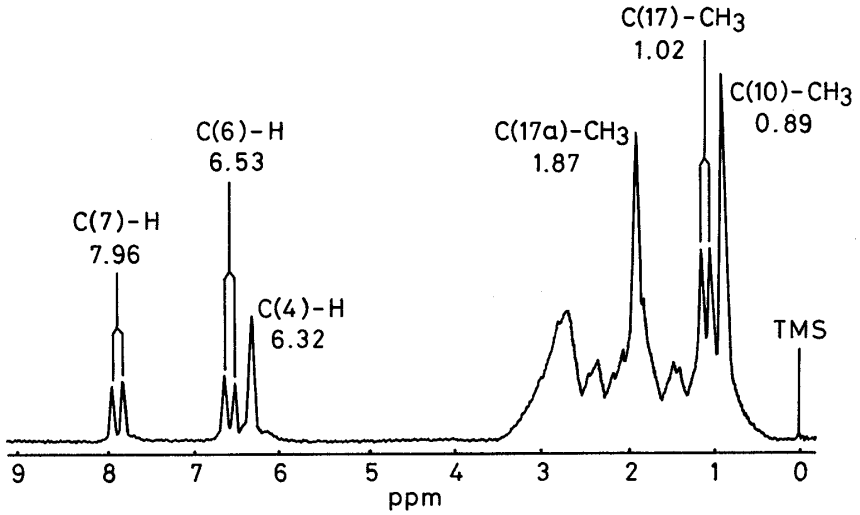

Fig. 3. ^{1}H-NMR Spectrum of χ-592 in a 1:2 Mixture of Sulfuric Acid-d_2 and Methanol-d_4

4a (40 mg) was dissolved in a 1:2 (v/v) mixture of D_2SO_4 and CD_3OD (0.5 ml).

ing the positive charge distribution in terms of the canonical structures of the hydroxyalkatetraenyl cation (**4b**), it is reasonable that C(7)-H is more deshielded than C(4)-H and C(6)-H. Furthermore, the spectral data for **4b** were in agreement with those[5] for the similar cation (**5b**) produced from testosterone (**7**), the structure of which was established previously by ^{1}H- and ^{13}C-NMR studies.[3d,e] Thus, χ-592 was identified as the hydroxyalkatetraenyl cation (**4b**).

As described above, the intermediary species, χ-427, was produced, accompanied with χ-284, by dissolving **1** or **2a** in 97% sulfuric acid. However, no product directly derived from χ-427 could be isolated because pouring the reaction mixture into excess ice-water resulted in the conversion of χ-427 to χ-592.

Though χ-592 is formed from **1** *via* **2b** and χ-427, the oxidation state of χ-592 (**4b**) is the same as that of **2b**. Therefore, an oxidation step is not involved in the formation of χ-592 from **2b**. The chemical reactions responsible for the formation of χ-592 from **2b** are thus assumed to be dehydrations, methyl migrations and acid-base-catalyzed double bond isomerizations, as shown in Chart 3.

In order to investigate the structure of χ-427, the effect of the acid strength on the conversion of χ-427 to χ-592 was examined. A solution of χ-427, prepared by dissolving **1** in 97% sulfuric acid, was diluted with excess sulfuric acid at various concentrations and the absorption intensities of the resultant solutions at 427 and 592 nm were measured. The results

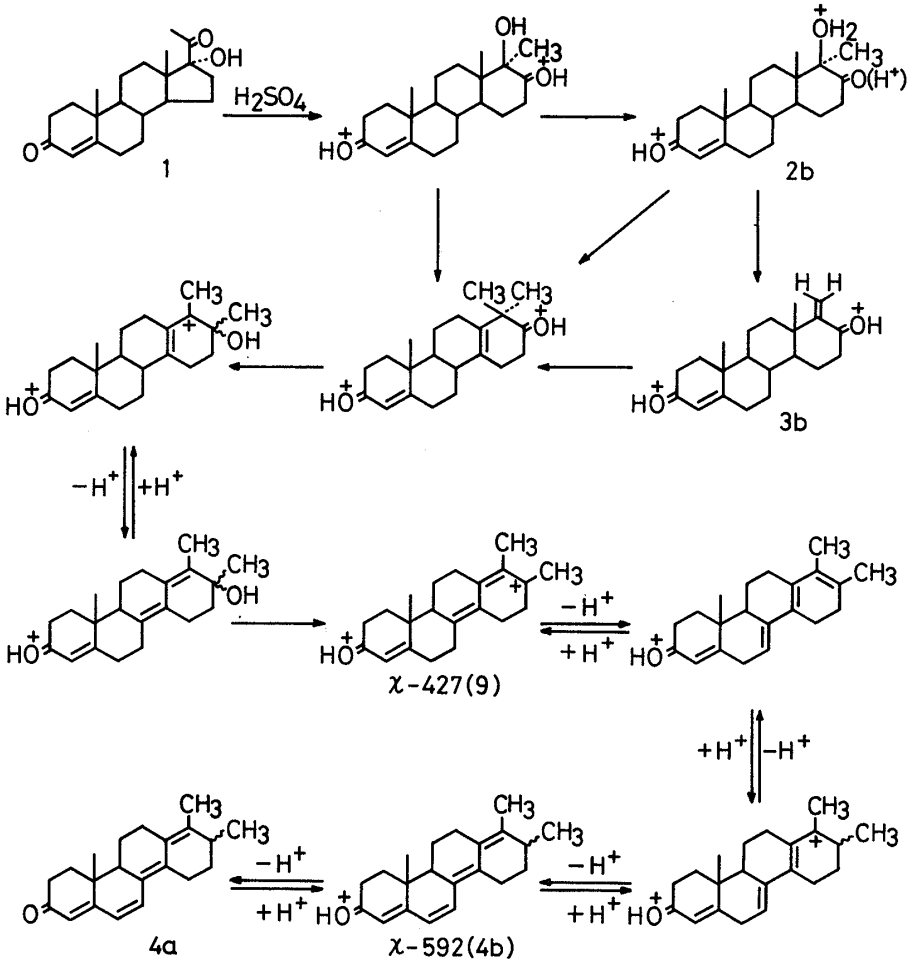

Chart 3. Proposed Mechanism of the Color and Fluorescence Reaction of 17α-Hydroxyprogesterone with Sulfuric Acid

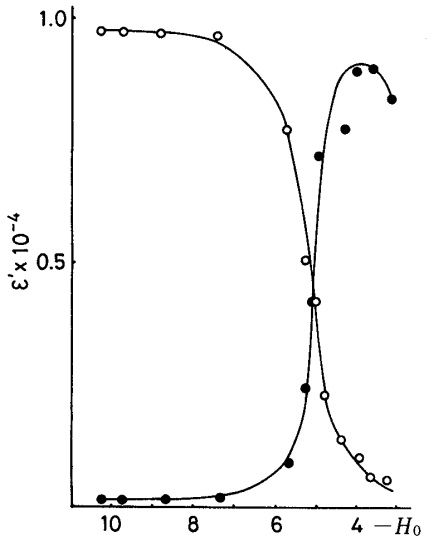

Fig. 4. Effect of Acid Strength on the Conversion of χ-427 to χ-592

A 97% H_2SO_4 solution of **1** (0.5 mg/ml) was heated at 60 °C for 45 min. The solution (500 μl) was then diluted with H_2SO_4 (5 ml) of various concentrations and the absorbance at 427 (○) or 592 nm (●) was measured after 10 min at room temperature.

obtained are shown in Fig. 4, where Hammett's acidity function (H_0) is used as an index of the acid strength of the resultant solutions.

Though χ-427 was stable in sulfuric acid where the $-H_0$ value was above 9, it became less stable and its conversion to χ-592 increased with decrease in the acid strength. In general,

dehydration and subsequent methyl migration occur faster with increase in the acid strength of the medium. On the other hand, the optimal acid strength for acid-base-catalyzed isomerization of a double bond depends on the pK_a value of the intermediate cation. Therefore, the reaction responsible for the conversion of χ-427 to χ-592 is assumed to be acid-base-catalyzed double bond isomerization. An alkyl cation is not stable even in concentrated sulfuric acid,[6] and has no absorption in the visible region.[7] An alkenyl cation, in general, is rather stable in concentrated sulfuric acid,[6] but shows an absorption maximum at 300 nm.[7] On the other hand, alkadienyl cations are known to show an absorption maximum at around 400 nm,[7] and some of them are stable in acid.[8] Thus, χ-427 is tentatively assigned as the dication (**9**) which has the hydroxyalkenyl cation in ring A and the alkadienyl cation across rings C and D.

On the basis of the results obtained in the present and previous studies,[1b] the color and fluorescence reaction of **1** is concluded to proceed by the mechanism shown in Chart 3. D-Homoannulation of **1** occurs rapidly to give **2b**. Removal of the hydroxyl group at C(17a) of **2b** and subsequent double methyl migrations, followed by dehydration, afford the intermediary species, χ-427 (**9**). Dehydration of **2b** to **3b** also occurs competitively and **3b** is then converted slowly to χ-427. χ-427 is stable in concentrated sulfuric acid, but is converted to the chromo- and fluorophoric species, χ-592 (**4b**), by acid-base-catalyzed double bond isomerizations when the acid strength of the medium is decreased.

Experimental

General Methods——Infrared (IR) spectra were measured with a JASCO A-102 recording spectrometer. Mass spectral (MS) measurements were made on a JEOL JMS-D300 spectrometer. Absorption and fluorescence spectra were measured with a Shimadzu UV-220 recording spectrometer and a Hitachi MPF-3 fluorescence spectrometer, respectively. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-FX100 FT spectrometer at 100 MHz or a Hitachi R-20B spectrometer at 60 MHz with tetramethylsilane as an internal standard. For preparative thin-layer chromatography (TLC), silica gel (Wakogel B-5F) was used as an adsorbent.

Materials——17α-Hydroxyprogesterone (**1**) was purchased from Sigma Chemical Co. and used after recrystallization. Sulfuric acid (super special grade, 97.2% w/w) was obtained from Wako Pure Chemical Industries Ltd.

Measurement of the ^{1}H-NMR Spectrum of χ-592——A 1 : 2 mixture of D_2SO_4 and CD_3OD (0.5 ml) was added to a dried sample (40 mg) of **4a** and the mixture was shaken vigorously to form a homogeneous solution. The ^{1}H-NMR spectrum of the solution was recorded at 35 °C using tetramethylsilane in a capillary as a reference.

Isolation of 17ξ,17a-Dimethyl-18-nor-D-homoandrosta-4,6,8(14),13(17a)-tetraen-3-one (4a)——A mixture of sulfuric acid (97%, 3 ml) and **1** (150 mg) was shaken vigorously to form a homogeneous solution, which was heated at 60 °C for 45 min. The reaction mixture was diluted with ethanol (6 ml) under ice-cooling to give a deep purple solution. The colored solution was gradually poured into excess ice-water under vigorous stirring and extracted with ethyl acetate (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 (9 : 1, × 2). Elution of the adsorbent corresponding to the yellow spot of *Rf* 0.52 with chloroform gave **4a** as a red-brown oil. λ_{max}^{EtOH} nm (ε): 400 (20700). High resolution MS *m/e* (M^+) Calcd for $C_{21}H_{26}O$: 294.1983, Found: 294.1974. MS *m/e*: 294 (M^+). IR ν_{max}^{neat} cm^{-1}: 1660 (C=O), 1560 (C=C). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.00 (3H, s, C(10)-CH_3), 1.07 (3H, d, J=7 Hz, C(17)-CH_3), 1.79 (3H, s, C(17a)-CH_3), 5.73 (1H, s, C(4)-H), 6.08 (1H, dd, J=9 Hz, C(6)-H), 6.88 (1H, d, J=9 Hz, C(7)-H).

Acknowledgement We are indebted to the staff of the Center for Instrumental Analysis, Hokkaido University, for the measurements of ^{1}H-NMR spectra and MS. A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References and Notes

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5) The absorption and fluorescence maxima of **5b** in a 2 : 3 mixture of sulfuric acid and ethanol are at 600 nm ($\varepsilon =$ 45800) and 615 nm, respectively. ^{1}H-NMR of **5b** (in 53% D_2SO_4) δ: 1.02 (3H, s, C(10)-CH_3), 2.11 (3H, s, C(17)-CH_3), 6.02 (1H, s, C(4)-H), 6.43 (1H, d, $J=8$ Hz, C(6)-H), 7.60 (1H, d, $J=8$ Hz, C(7)-H).
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