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^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectra of Testosterone and 17β-Hydroxyandrosta-4,6-dien-3-one in Sulfuric Acid[1)]

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The behavior of testosterone (I) and 17β-hydroxyandrosta-4,6-dien-3-one (VII) in sulfuric acid was investigated by absorption and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopic studies. I and VII were demonstrated to give initially the diprotonated species, II and VIII, respectively and then the dication (χ-300, III), which is the intermediate in the color and fluorescence reaction of I with strong acids. The conjugate base of χ-300, 17-methyl-18-norandrosta-4,8(14),13(17)-trien-3-one (IV), was isolated from the reaction mixture of VII with 97% sulfuric acid. Thus, the chemical structure of χ-300 was fully elucidated and the reaction mechanism previously proposed was confirmed.

Keywords——testosterone; 17β-hydroxyandrosta-4,6-dien-3-one; sulfuric acid; ^{1}H-NMR; ^{13}C-NMR; unsaturated ketosteroids; steroidal carbocation; dication; cyclopentenyl cation; 17-methyl-18-norandrosta-4,8(14),13(17)-trien-3-one

During the course of the studies of this series, the mechanism of the color and fluorescence reactions of steroidal estrogens with sulfuric acid was investigated.[1b,2)] It was clarified that the chromophores and fluorophores thus formed are steroidal carbocations.[1b,2)] Although this sort of reaction has been well studied with unsaturated ketosteroids such as testosterone,

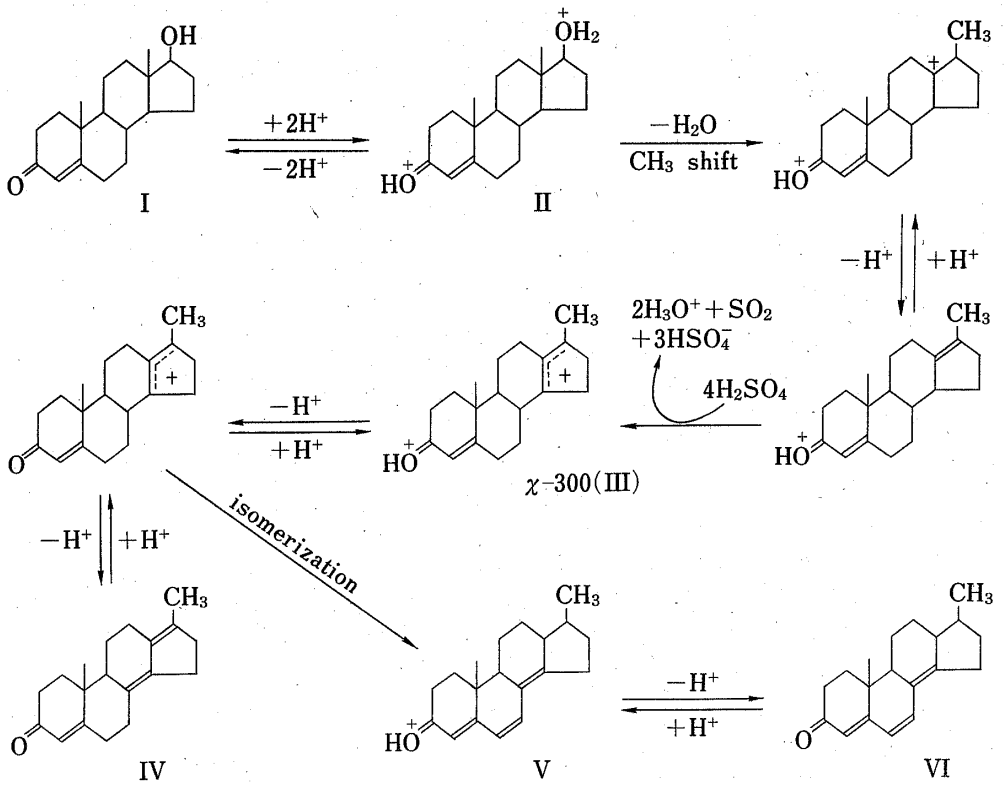


Chart 1. Primary Process of the Color and Fluorescence Reaction of Testosterone (I) with Sulfuric Acid

progesterone, and corticoids,[3-10] the mechanisms remain largely unknown. Recently, we pointed out that testosterone (I), 17β-hydroxyandrost-4-en-3-one, reacted with sulfuric acid to give an intermediate having a maximum absorption at 300 nm (abbreviated as χ-300) which then transformed to the chromo- and fluorophore, the hydroxyalkatrienyl cation (V), as shown in Chart 1.[5] It was also suggested that the intermediary χ-300 is the dication (III).[4] We observed the time-course of the behavior of I and 17β-hydroxyandrosta-4,6-dien-3-one (VII) in sulfuric acid by using ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, then we isolated the reaction product, and finally we elucidated its chemical structure. This paper presents unambiguous evidence supporting the assignment of χ-300 as the dication (III).

Results and Discussion

The chemical species χ-300 (λ_{max}: 300 nm, ε=26,000) was rapidly formed when VII was dissolved in 97% sulfuric acid, as is the case with I.[4] The formation of χ-300, however, was slow in sulfuric acid of lower concentration. A maximum absorption at 292 nm (ε=16,700) appeared immediately after I was dissolved in 90% sulfuric acid and it gradually shifted to 300 nm (Fig. 1a). In contrast, VII first gave a maximum at 352 nm (ε=32,200) which then blue-shifted to 300 nm, as shown in Fig. 1b. Protonation of the carbonyl oxygen leads to an α,β- or $\alpha,\beta,\gamma,\delta$-unsaturated ketone to give a maximum absorption at about 290 or 350 nm, respectively.[4,6,14] The chemical species initially formed from I and VII in 90% sulfuric acid may, therefore, be the dications II and VIII, respectively.

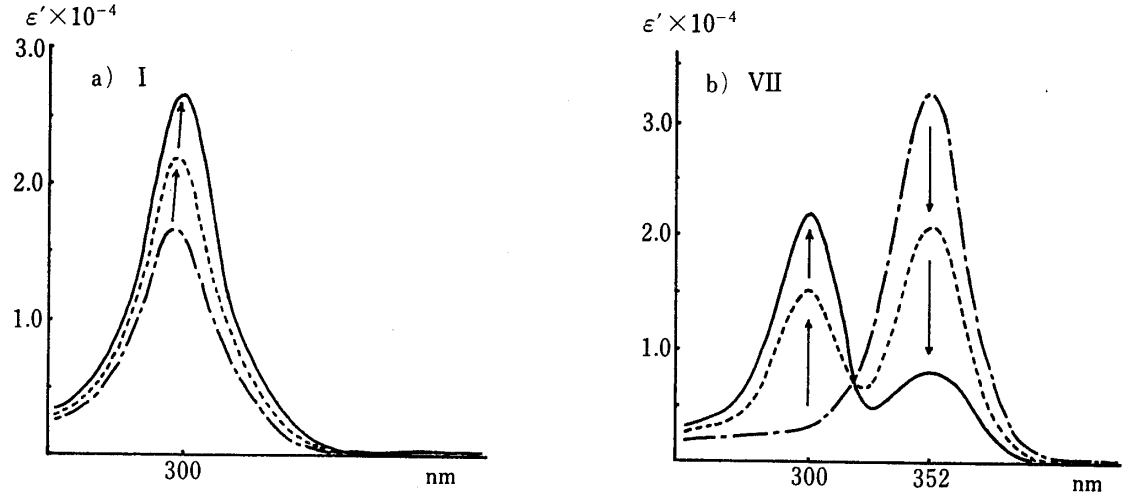


Fig. 1. Absorption Spectra of Testosterone (I) and 17β-Hydroxyandrosta-4,6-dien-3-one (VII) in 90% Sulfuric Acid

——: 5 min, - - -: 30 min, ——: 50 min.

In the ^{1}H-NMR spectrum (Fig. 2) at 10 min after dissolving I in 90% sulfuric acid, the signals due to C(4)-H and C(17α)-H were, in contrast to those[13a] of I in $CDCl_3$, markedly shifted to lower field, δ 6.82 and 4.65, respectively. This may indicate that the chemical species showing a maximum absorption at 292 nm is the dication II, in which the oxygens of the carbonyl group at C(3) and the hydroxyl group at C(17) are both protonated. Similarly, the ^{1}H-NMR spectrum[13b] of VII (Fig. 3) indicated the initial formation of the chemical species VIII which gave a maximum absorption at 352 nm. The structures of II and VIII are consistent with those of the cations XI and XII already reported[14] on the basis of the ^{1}H-NMR data (Chart 2 and Table I).

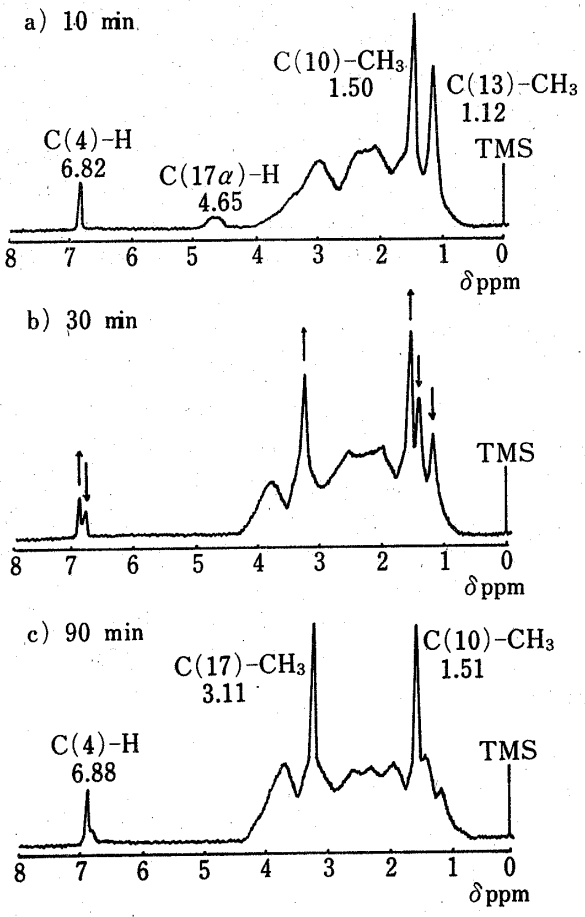


Fig. 2. ^{1}H-NMR Spectra of Testosterone (I) in 90% Sulfuric Acid with the Passage of Time[13a)]

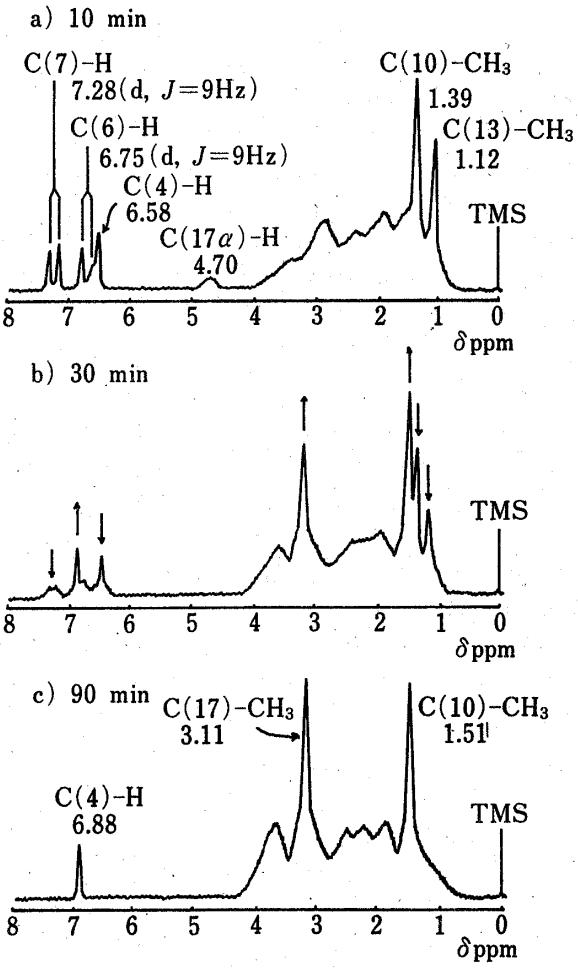
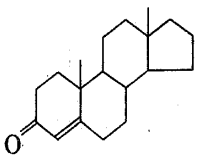


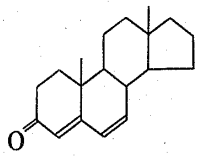
Fig. 3. ^{1}H-NMR Spectra of 17β-Hydroxyandrosta-4,6-dien-3-one (VII) in 90% Sulfuric Acid with the Passage of Time[13b)]

XIII

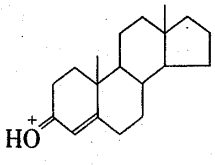
C-1 247.1
C-2 155.4
in FSO_3H-SbF_5-SO_2ClF



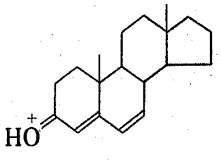
IX



X



XI



XII

Chart 2

Table I. ^{1}H-NMR Data for Unsaturated Ketosteroids (IX and X) in $CDCl_3$ and 80% Sulfuric Acid

	In 80% H_2SO_4	In $CDCl_3$
	XI (294 nm, ε=14100)	IX
C(13)–CH_3	1.03 δ ppm	0.76 δ ppm
C(10)–CH_3	1.55	1.20
C(4)–H	6.88	5.77
	XII (360 nm, ε=32500)	X
C(13)–CH_3	1.05	0.80
C(10)–CH_3	1.46	1.12
C(4)–H	6.68	5.67
C(6)–H	6.82 (d, J=9 Hz)	6.13
C(7)–H	7.47 (d, J=9 Hz)	6.13

In the ^{1}H-NMR spectra of I or VII in 90% sulfuric acid, the signal of II or VIII, respectively, decreased with time, and new signals due to C(4)-H and C(17)-CH_3 of χ-300 then appeared at 6.88 and 3.11 ppm, respectively (Figs. 2 and 3). The spectra given by both I and VII finally became identical with that[4] given by the above-mentioned χ-300. The changes of the ^{1}H-NMR spectra with time were in good accord with those observed for the absorption spectra (Fig. 1), and thus the formation of χ-300 from VII was indicated to be more rapid than that from I. These results show that both I and VII in highly concentrated sulfuric acid are transformed to the common chemical species χ-300, in spite of the different processes of its formation. The ^{13}C-NMR spectrum is, generally, preferable to ^{1}H-NMR for obtaining direct evidence of chemical structure. Thus, a solution of II or VIII, which was prepared by dissolving I or VII, respectively, in 73% sulfuric acid was studied by ^{13}C-NMR spectroscopy, and gave data substantiating the indicated structures of these cations, as expected (Table II).

Table II. ^{13}C-NMR Data for Testosterone (I) and 17β-Hydroxyandrosta-4,6-dien-3-one (VII) in $CDCl_3$ and 73% Sulfuric Acid

	I		VII	
	In 73% H_2SO_4	In $CDCl_3$	In 73% H_2SO_4	In $CDCl_3$
C-3	210.8 δ ppm	199.4 δ ppm	208.4 δ ppm	199.6 δ ppm
C-4	120.3	123.8	118.3	123.8
C-5	205.4	171.3	191.1	163.8
C-6			128.8	128.0
C-7			159.1	140.5
C-17	84.8	81.5	83.8	81.3

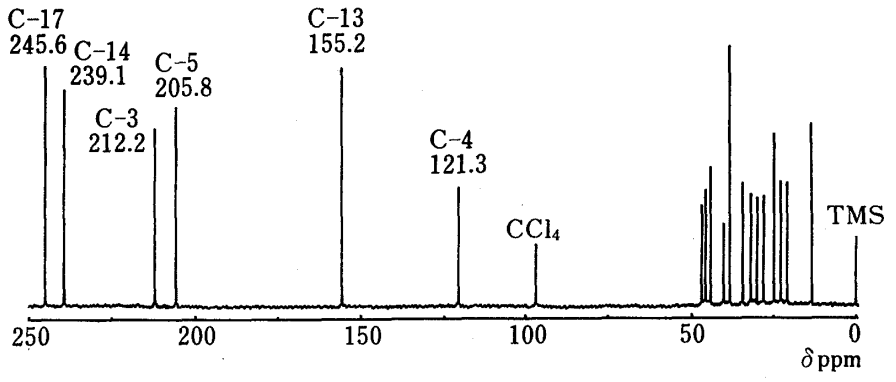


Fig. 4. ^{13}C-NMR Spectrum of Testosterone (I) or 17β-Hydroxyandrosta-4,6-dien-3-one (VII) in 97% Sulfuric Acid

On the other hand, a solution of χ-300, which was prepared by dissolving I in 97% sulfuric acid, gave a ^{13}C-NMR spectrum (Fig. 4) identical with that given by a solution of VII in the same acid. In this spectrum, the signals due to C(3), C(4), and C(5) appeared at δ 212.2, 121.3, and 205.8 respectively, and additional signals were observed at δ 245.6, 239.1, and 155.2. The latter three signals coincide very closely with those given by the cyclopentenyl cation (XIII, Chart 2),[11] which may thus represent a partial structure of χ-300. Although complete assignment of ^{13}C-NMR signals given by χ-300 was not achieved, the characteristic signals mentioned above suggest that χ-300 (III) is a dication having the hydroxylalkenyl cation moiety of C(3)-C(4)-C(5), on the one hand, and the cyclopentenyl cation moiety of C(17)-C(13)-C(14), on the other.

When a mixture of VII and 97% sulfuric acid was carefully poured over ice and extracted with ether, preparative thin-layer chromatography (TLC) of the extract gave a light-yellow oil, *m/e*: 268(M^+), λ_{max}^{EtOH}: 244 nm (ε=22,000), $\nu_{C=O}$: 1670 cm^{-1}, in about 18% yield. Since the oil showed a single absorption maximum and its molar absorptivity was larger than that given by a normal α,β-unsaturated ketone such as testosterone (ε=17,800), it may be a 4-en-3-one steroid with a conjugated diene system. In the ^{1}H-NMR spectrum, on the other hand, no signal due to a vinylic proton was observed other than that due to C(4)-H, but the signal due to C(17)-CH_3 as a vinylic methyl group appeared at δ 1.74. These spectral data suggest that the oil is 17-methyl-18-norandrosta-4,8(14),13(17)-trien-3-one (IV), the conjugate base of the dication (III). Moreover, the ^{13}C-NMR spectrum of this oil gave seven signals due to the sp^2 carbons, C(3), C(4), C(5), C(8), C(13), C(14), and C(17). It may reasonably be concluded from these results that χ-300 is the dication (III) and the reaction mechanism proposed previously (Chart 1)[4,5] is thus confirmed by this study.

On the basis of the facts described below, the formation of χ-300 (III) from VII in highly concentrated sulfuric acid is assumed to proceed by the mechanism shown in Chart 3. Since 17α-alkyl-17β-hydroxyandrosta-4,6-dien-3-one in sulfuric acid is transformed to 17α-alkyl-17β-methyl-18-norandrosta-4,6,8(14)-trien-3-one,[8] the trienone VI may be similarly produced from VII by a series of processes such as protonation, dehydration, and rearrangement of methyl group. In contrast to the stability of XI and XII in 97% sulfuric acid, the cation **V** once formed was reported to be so unstable in such a highly concentrated acid that it was further protonated and easily deconjugated to give χ-300 (III).[14]

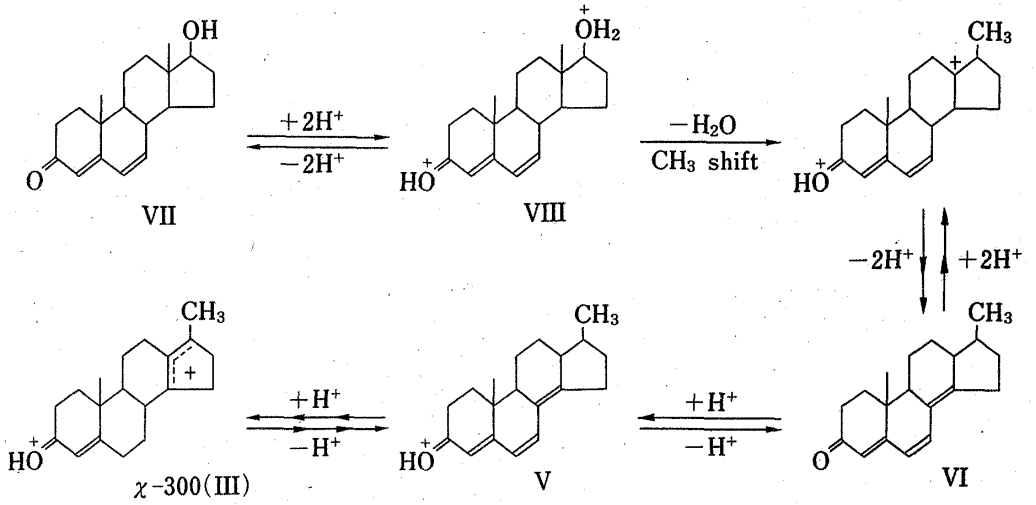


Chart 3. Reaction Mechanism of 17β-Hydroxyandrosta-4,6-dien-3-one (VII) with Sulfuric Acid

That the dienone VII can, as described above, give χ-300 (III) more rapidly than testosterone (I) in 90% sulfuric acid (Fig. 1) may be explained on the basis of the proposed reaction mechanism, since the oxidative process is unnecessary for VII, in contrast to I.

Experimental

General Methods——Absorption spectra were measured with a Shimadzu UV-220 recording spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-FX 100 FT spectrometer with tetramethylsilane as an internal standard at 100 and 25.0 MHz, respectively; the former spectra were also recorded on a Hitachi R-20-B spectrometer at 60 MHz. Chemical shifts are given in δ (ppm) values. Mass spectra (MS) measurement was run on a JEOL JMS-D-300 spectrometer. Infrared (IR) spectra were taken on a JASCO A-102 spectrometer. For preparative TLC, silica gel (Wakogel B5F) was used as an adsorbent.

Materials——Testosterone (I) was a commercial product and was used after recrystallization. 17β-Hydroxyandrosta-4,6-dien-3-one (VII) was prepared according to the reported method.[12] Sulfuric acid (super special grade, 97.2% w/w, Wako Pure Chem. Ind. Ltd.) and sodium 3-(trimethylsilyl)propane sulfonate (Merck) were obtained commercially and used without purification.

Absorption Spectra in Sulfuric Acid——Sulfuric acid (5 ml) was added to a dried sample (40—60 μg) at room temperature. The mixture was shaken vigorously to form a homogeneous solution and measured on a spectrometer at 25°C.

NMR Spectra in Sulfuric Acid——A mixture of sulfuric acid (0.5 ml, 73, 90, or 97%) and a sample (40 mg) was shaken vigorously to form a homogeneous solution. ^{1}H-NMR spectra were recorded at 35°C and ^{13}C-NMR spectra were obtained by the Fourier transform method at 35°C using external capillary tetramethylsilane as a reference. The deuterium signal of D_2O was used for a hetero nuclear lock in the latter NMR. The solvent effect of sulfuric acid on a chemical shift was shown to be within δ 0.15 and 0.7 for ^{1}H and ^{13}C, respectively, based on the measured difference between external capillary tetramethylsilane and sodium 3-(trimethylsilyl) propane sulfonate in 0—97% sulfuric acid.

Isolation of the Conjugate Base (IV) of χ-300 (III)——A mixture of sulfuric acid (97%, 5 ml) and VII (300 mg) was shaken vigorously to form a homogeneous solution, which was allowed to stand at room temperature for 20 min. The reaction mixture was gradually poured over ice-water (400 ml) and extracted with ether (200 ml × 3). The ether layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue (285 mg) thus obtained was subjected to preparative TLC and developed with benzene–acetone (4: 1). Elution of the adsorbent corresponding to the spot of *Rf* 0.54 with ether gave 17-methyl-18-norandrosta-4,8(14),13(17)-trien-3-one (IV) as a light-yellow oil (51 mg). MS *m/e*: 268 (M^+, base peak). UV λ_{max}^{EtOH} nm (ε): 244 (22000). IR ν_{max}^{Nujol} cm^{-1}: 1670 (C=O), 1610 (C=C). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.10 (3H, s, C(10)–CH_3), 1.74 (3H, s, C(17)–CH_3), 5.78 (1H, s, C(4)–H). ^{13}C-NMR (10% solution in $CDCl_3$) δ: 121.3 (C(8)), 123.5 (C(4)), 133.7, 136.6, and 142.2 (C(13), C(14), or C(17)), 172.1 (C(5)), 199.3 (C(3)).

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References and Notes

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13) *a*) I in $CDCl_3$ δ: C (4)–H (5.72), C (17α)–H (3.64), C (10)–CH_3 (1.20), C (13)–CH_3 (0.79); *b*) VII in $CDCl_3$ δ: C (6)– and C (7)–H (6.11), C (4)–H (5.68), C (17α)–H (3.69), C (10)–CH_3 (1.13), C (13)–CH_3 (0.85).
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