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D-Homoannulation in the Color and Fluorescence Reaction of 17α-Hydroxyprogesterone with Sulfuric Acid[1)]

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Dissolution of 17α-hydroxyprogesterone (**1**) in 80% sulfuric acid gave two D-homosteroids through C(13)–C(17) bond migration, 17aβ-hydroxy-17aα-methyl-D-homoandrost-4-ene-3,17-dione (**2**) and 17a-methylene-D-homoandrost-4-ene-3,17-dione (**3**), in 53 and 9% yields, respectively. The latter product was also obtained in 73% yield from the reaction of **1** with 90% sulfuric acid. Proton nuclear magnetic resonance (^{1}H-NMR) studies of the acid solutions revealed that the D-homoannulation of **1** to **2** occurs rapidly even in 80 or 90% sulfuric acid, and that **2** is dehydrated rapidly to **3** in 90% sulfuric acid. The D-homoannulation was demonstrated to be an initial step in the color and fluorescence reaction of **1** with sulfuric acid.

Keywords——17α-hydroxyprogesterone; color-fluorescence reaction; sulfuric acid; D-homoannulation; ^{1}H-NMR analysis; mechanism

A number of organic compounds develop color and fluorescence when they come into contact with a strong acid. These color and fluorescence reactions have been widely utilized for the determination of these compounds, especially for the quantitation of steroidal compounds in biological fluids and in pharmaceutical preparations, though their mechanisms have remained unknown.

In this series of studies, the reaction mechanism of steroidal estrogens,[2)] testosterone[1b,3)] and progesterone[4)] have been elucidated, and in all cases the chromo- and fluorophores were shown to be steroidal carbocations produced by protonation, dehydration, rearrangement and oxidation.

This paper deals with the D-homoannulation of 17α-hydroxyprogesterone (**1**) which occurs at the early stage of the color and fluorescence reaction of **1** with sulfuric acid.

Results and Discussion

Many steroids produce color and fluorescence when they are dissolved in concentrated sulfuric acid, followed by dilution with water or ethanol. Such is the case for estrogens[2)] and testosterone,[3)] and also for **1**.

When **1** was dissolved in 97% sulfuric acid at room temperature, two chemical species were formed. One showed an absorption maximum at 284 nm and the other at 427 nm with fluorescence at 472 nm (abbreviated as χ-284 and χ-427, respectively). The latter species, χ-427, gradually increased with the passage of time and the rate of its formation increased with increase in temperature. Upon dilution of the reaction mixture with two volumes of ethanol, a new chromo- and fluorophoric species showing an absorption maximum at 592 nm with fluorescence at 614 nm (abbreviated as χ-592) was produced with the disappearance of χ-427, as shown in Fig. 1.

On the other hand, when **1** was dissolved in 80 or 90% sulfuric acid at room temperature,

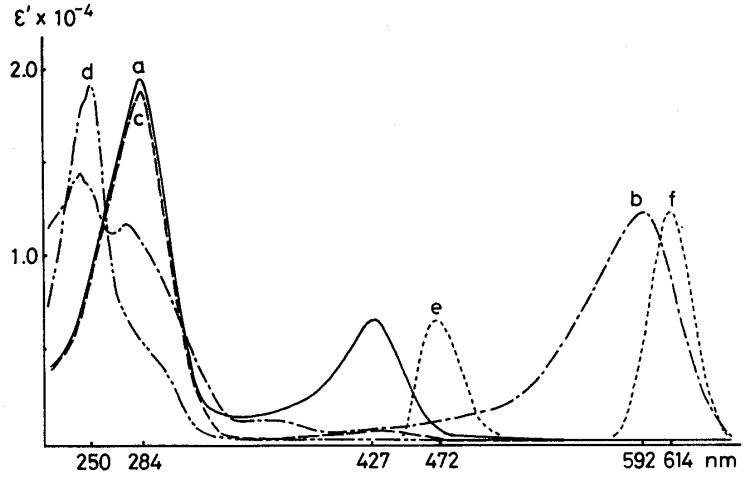

Fig. 1. Color and Fluorescence Reaction of 17α-Hydroxyprogesterone (**1**) in the Sulfuric Acid–Ethanol System

Each spectrum was measured at 5 min after preparing the solution at room temperature. ε′: Apparent molar absorptivity. a) Compound **1** (52 μg) was dissolved in 97% H_2SO_4 (5 ml). Absorption spectrum. b) The 97% H_2SO_4 solution of **1** (2 ml) was poured into ethanol (4 ml). Absorption spectrum. c) Compound **1** (52 μg) was dissolved in 90% H_2SO_4 (5 ml). Absorption spectrum. d) The 90% H_2SO_4 solution of **1** (2 ml) was poured into ethanol (4 ml). Absorption spectrum. e) and f) Fluorescence spectra of the sulfuric acid–ethanol solutions of **1** described c) and d) with excitation at 430 and 589 nm, respectively.

χ-427 was scarcely formed, though χ-284 was produced to the same extent as with 97% sulfuric acid. In addition, no formation of χ-592 was observed on diluting acid solutions containing no χ-427 with two volumes of ethanol. These results suggested that χ-427 is an essential intermediate in the formation of χ-592.

In order to elucidate the mechanism of the early stage of the color and fluorescence reaction of **1** with sulfuric acid, isolation of the products from the reactions of **1** with 80 and 90% sulfuric acid, and proton nuclear magnetic resonance (^{1}H-NMR) studies of the acid solutions of **1** were carried out as follows.

Compound **1** was dissolved in 80% sulfuric acid and allowed to stand at room temperature for 10 min, then the reaction mixture was poured into excess ice water, extracted with ethyl acetate and separated by preparative thin-layer chromatography (TLC) to give **2** and **3** in 53 and 9% yields, respectively. On the other hand, **3** was isolated in 73% yield without any detectable amount of **2** from the reaction of **1** with 90% sulfuric acid. The mass spectrum (MS) of **2** showed a molecular ion peak at *m/e* 330, which is consistent with the molecular weight of **1**, indicating that **2** is an isomer of **1**. Compound **2** was shown to have a hydroxyl, and both saturated and unsaturated carbonyl groups by the presence of bands at 3484, 1713 and 1678 cm^{-1} in its infrared (IR) spectrum. Since the ultraviolet (UV) spectrum exhibited an absorption maximum at 236 nm ($\varepsilon = 19000$) and the ^{1}H-NMR spectrum showed a singlet signal at 5.74 ppm due to the olefinic proton at C(4), the 4-en-3-one structure remains intact in **2**. The hydroxyl group was shown to be tertiary by the absence of a signal due to a proton on the carbon bearing the hydroxyl group. The ^{1}H-NMR spectrum also showed three singlet methyl signals at 0.78, 1.18 and 1.33 ppm. The former two are due to angular methyl groups at C(13) and C(10), and the latter one is assignable not to an acetyl group but to a methyl group on the carbon bearing the hydroxyl group. These results suggested that compound **2** is a D-homosteroid having a ketol moiety in ring D.

Several examples are known of the D-homoannulation of a series of pregnanes having 17α- or 17β-hydroxy-20-one systems under Lewis acid, alkaline or thermal conditions.[5-7] The Lewis acid-catalyzed D-homoannulation of 17α-hydroxy-20-one systems (**4**) affords 17α-

hydroxy-17β-methyl-17a-ones (**5**) resulting from C(16)–C(17) bond migration. In contrast, the alkaline-catalyzed rearrangement (acyloin rearrangement) of **4** gives 17aβ-hydroxy-17aα-methyl-17-ones (**6**) as a major product resulting from C(13)–C(17) bond migration. With the latter rearrangement, Goutarel *et al.*[8] obtained a mixture of 17aβ-hydroxy-17aα-methyl-17-

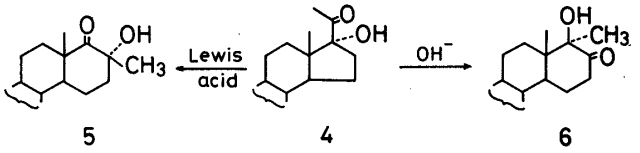

Chart 1

one (**6**) and its epimer at C(17a) from **1**. The melting point and spectral data of the former D-homosteroid were in good agreement with those of **2**. Thus, compound **2** was identified as 17aβ-hydroxy-17aα-methyl-D-homoandrost-4-ene-3,17-dione. Though the configuration at C(17a) of **2** was tentatively assigned by Goutarel *et al.*, it was confirmed by the IR band at 3484 cm^{-1}, indicating intramolecular hydrogen bonding between the equatorial hydroxyl group at C(17aβ) and the oxo group at C(17). Such hydrogen bonding is not possible in the epimer of **2** which has the axial hydroxyl group at C(17aα).[7b,9]

Though no example has previously been reported of Brönsted acid-catalyzed D-homoannulation of pregnanes having a 17-hydroxy-20-one systems, it was demonstrated by this study that D-homoannulation of **1** occurs in sulfuric acid to give **2** as a result of C(13)–C(17) bond migration as shown in Chart 2.

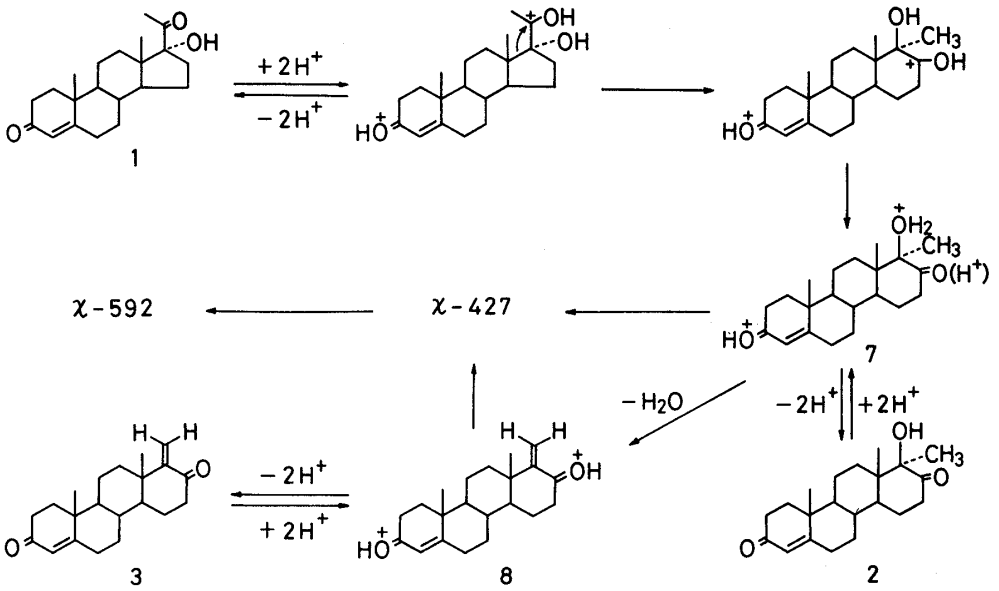

Chart 2

Compound **3** was also obtained by dissolving **2** in 90% sulfuric acid. The MS of **3** showed a molecular ion peak at *m/e* 312, indicating that **3** is a dehydration product of **2**. The IR spectrum showed no absorption due to a hydroxyl group but contained two strong bands at 1696 and 1678 cm^{-1} due to unsaturated carbonyl groups. In the ^{1}H-NMR spectrum, the methyl signal at C(17a) of **2** was replaced by two doublet signals due to olefinic protons. The coupling constant (J = 2.0 Hz) between the olefinic protons proves that **3** has an *exo* methylene group at C(17a). From these data, compound **3** was concluded to be 17a-methylene-D-

homoandrost-4-ene-3,17-dione. The structure of **3** was finally confirmed by comparing its melting point and spectral data with those of an authentic sample prepared by a different method.[10] Although **3** has an enone moiety in both ring A and ring D, its molar absorptivity ($\varepsilon = 19800$) at 238 nm is only slightly higher than that ($\varepsilon = 19000$) of **2** having the moiety in ring A. This suggests that coplanarity of the enone in ring D is partially prevented in **3**. The coplanarity is allowed in a half-boat conformation of ring D, but this conformation is prevented because of the steric hindrance between hydrogen at C(12) and the methylene at C(17a). Therefore, ring D of **3** seems to exist in a half-chair conformation, in which coplanarity of the enone is less favorable. This view is supported by the C=O stretching frequency (1696 cm^{-1}) of the enone which is somewhat higher than that normally found for an enone.

The reaction of **1** with sulfuric acid was further investigated by ^{1}H-NMR studies of the acid solutions of **1**, **2** and **3**. Both **1** and **2** gave the same ^{1}H-NMR spectrum (Fig. 2) at 5 min after dissolving them in 80% sulfuric acid. The signal pattern was similar to that given by **2** in $CDCl_3$ but not to that given by **1** in $CDCl_3$, though all the signals were shifted to lower field compared with those of **2**. Thus, this spectrum indicated that **1** and **2** are present as the protonated form (**7**) of **2** in the acid solution.

On the other hand, the ^{1}H-NMR spectrum of 90% sulfuric acid solution of **3** (Fig. 3) showed a signal pattern similar to that of **3** in $CDCl_3$, except that all the signals were shifted to lower field compared with those of **3**. The shifts of the signals from the values found for **3** were in the following order: olefinic protons of the *exo* methylene at C(17a) (1.77 and 1.67) > C(4)-H (1.04) > C(13)-CH_3 (0.30) > C(10)-CH_3 (0.27). These data indicated that **3** is present as the protonated form (**8**) in the acid. Since dissolution of **1** or **2** in 90% sulfuric acid resulted in the same spectrum as that of **8**, conversion of **1** and **2** to **3** was shown to occur rapidly in 90% sulfuric acid through D-homoannulation and/or dehydration. These results were in accordance with the products analysis described above.

In addition to **2** and **3**, pregna-4,16-diene-3,20-dione (**9**) and 17β-methyl-18-nor-17α-pregna-4,13-diene-3,20-dione (**10**) were thought to be possible products, but no evidence for their formation was obtained in the reaction of **1** with 80 or 90% sulfuric acid. Further, authentic **9** and **10** showed different absorptions (**9**: $\lambda_{max} = 290$ nm, $\varepsilon = 26200$. **10**: $\lambda_{max} = 288$ nm, $\varepsilon = 16500$) and ^{1}H-NMR spectra (Fig. 4a, b) from those of **2** and **3** in 90% sulfuric acid.

In relation to the mechanism of the color and fluorescence reaction of **1**, the behavior of **1**, **2** and **3** in sulfuric acid was investigated spectroscopically. As described above, χ-284 was formed when **1** was dissolved in 80 or 90% sulfuric acid. Since dissolution of each of **2** and **3** in

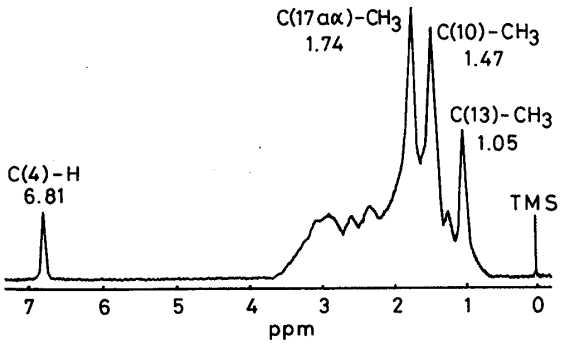

Fig. 2. ^{1}H-NMR Spectrum of **1** in 80% Sulfuric Acid

Compound **1** (40 mg) was dissolved in 0.5 ml of 80% H_2SO_4.

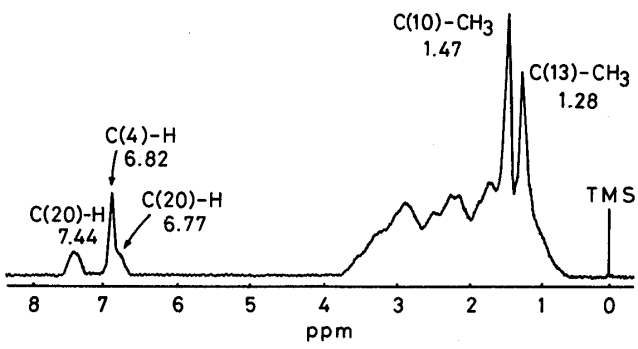

Fig. 3. ^{1}H-NMR Spectrum of **3** in 90% Sulfuric Acid

Compound **3** (40 mg) was dissolved in 0.5 ml of 90% H_2SO_4.

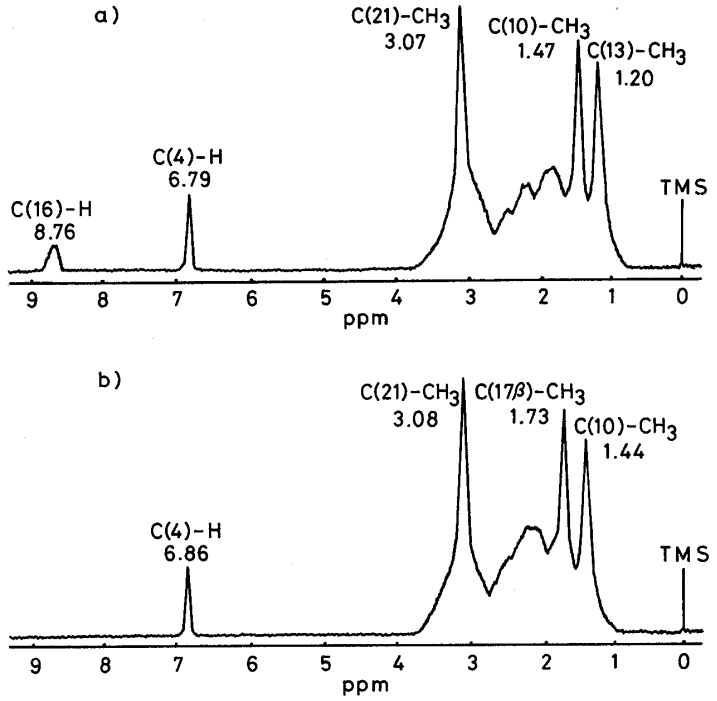

Fig. 4. ^{1}H-NMR Spectra of **9** and **10** in 90% Sulfuric Acids

a) Compound **9** (40 mg) was dissolved in 0.5 ml of 90% H_2SO_4. b) Compound **10** (40 mg) was dissolved in 0.5 ml of 90% H_2SO_4.

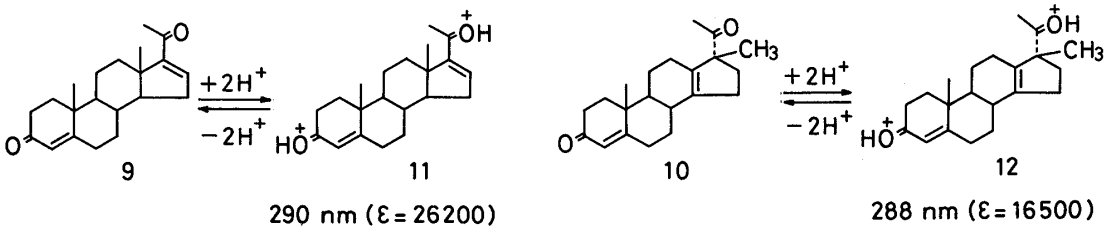

Chart 3

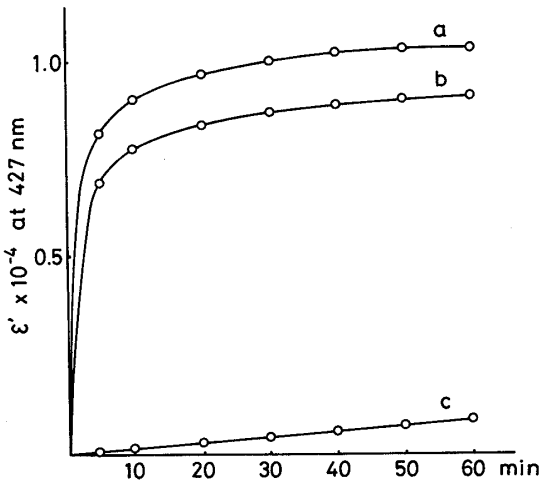

Fig. 5. The Time Course of the Formation of χ-427 from **1**, **2** and **3** in 97% Sulfuric Acid

Compound **1** (56 μg), **2** (51 μg) or **3** (51 μg) was dissolved in 97% H_2SO_4 (5 ml) at room temperature. a) from **2**. b) from **1**. c) from **3**.

80 or 90% sulfuric acid also gave χ-284 immediately, the protonated forms (**7** and **8**) of **2** and **3** both contribute to the absorption at 284 nm. On the other hand, not only **1** but also **2** and **3** gave χ-427 as well as χ-284 when they were dissolved in 97% sulfuric acid. Figure 5 showed the time courses of the formation of χ-427 from **1**, **2** and **3**; χ-427 was produced in two phases

from **1** and **2**, smoothly in the first phase and slowly in the second phase, though its formation was somewhat greater from **2** than from **1**. On the other hand, **3** gave χ-427 slowly at a constant rate similar to that of the second phase given by **1** or **2**. These results indicated that χ-427 is formed from **1** by the pathway shown in Chart 1. D-Homoannulation of **1** occurs rapidly to give **7**, which transforms to χ-427. The conversion of **7** to χ-427 competes with dehydration of **7** to **8**. χ-427 is also formed slowly from **8** once this is produced. At the early stage of the color and fluorescnece reaction of **1** with sulfuric acid, such reactions may occur to give χ-427, which is the intermediate in the formation of the chromo- and fluorophoric species, χ-592. The chemical structures of χ-427 and χ-592 will be discussed in the subsequent paper.

Experimental

General Methods——All melting points were taken on a micro hot-stage apparatus and are corrected. IR spectra were measured with a JASCO A-102 recording spectrometer. MS measurements were run 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. ^{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 TLC, silica gel (Wakogel B-5F) was used as an adsorbent.

Materials——Sulfuric acid (super special grade, 97.2% w/w) was obtained from Wako Pure Chemical Industries Ltd. 17α-Hydroxyprogesterone (**1**) and pregna-4,16-diene-3,20-dione (**9**) were purchased from Sigma Chemical Co. and used after recrystallization. 17β-Methyl-18-nor-17α-pregna-4,13-diene-3,20-dione (**10**) was prepared according to the method of Schmitt *et al.*[11]

^{1}H-NMR Spectra in Sulfuric Acid——Sulfuric acid (80 or 90% w/w, 0.5 ml) was added to a dried sample (40 mg), and the mixture was shaken vigorously to obtain a homogeneous solution. The ^{1}H-NMR spectra of the solutions were recorded at 35 °C, with tetramethylsilane in a capillary as a reference.

Isolation of 17aβ-Hydroxy-17aα-methyl-D-homoandrost-4-ene-3,17-dione (2) and 17a-Methylene-D-homoandrost-4-ene-3,17-dione (3)——A mixture of 80% sulfuric acid (5 ml) and **1** (300 mg) was shaken vigorously to give a homogeneous solution and then allowed to stand at room temperature for 10 min. The reaction mixture 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 spot of *Rf* 0.29 with chloroform gave 159 mg of **2**, which was crystallized from acetone to afford colorless plates, mp 186—188 °C (lit.[8] 190 °C). *Anal.* Calcd for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.31; H, 9.15. λ_{max}^{EtOH} nm (ε): 236 (19000). MS *m/e*: 330 (M^+). IR (0.005 M, in CCl_4) cm^{-1}: 3484 (OH), 1713 (C=O), 1678 (conjugated C=O), 1620 (C=C). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.78 (3H, s, C(13)-CH_3), 1.18 (3H, s, C(10)-CH_3), 1.33 (3H, s, C(17aα)-CH_3), 3.82 (1H, s, C(17aβ)-OH), 5.74 (1H, s, C(4)-H). Another adsorbent corresponding to the spot of *Rf* 0.40 was treated in the same manner to give 26 mg of **3** as colorless needles, mp 148—150 °C (lit.[11] 158 °C). *Anal.* Calcd for $C_{21}H_{28}O_2$: C, 80.73; H, 9.03. Found: C, 80.46; H, 9.09. λ_{max}^{EtOH} nm (ε): 238 (19800). MS *m/e*: 312 (M^+). IR (0.005 M, in CCl_4) cm^{-1}: 1696, 1678 (conjugated C=O), 1619 (C=C). ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.98 (3H, s, C(13)-CH_3), 1.20 (3H, s, C(10)-CH_3), 5.10 (1H, d, J=2.0 Hz, *exo* methylene), 5.67 (1H, d, J=2.0 Hz, *exo* methylene), 5.78 (1H, s, C(4)-H). When a mixture of 90% sulfuric acid (7 ml) and **1** (500 mg) was treated in the same manner as described above, 343 mg of **3** was obtained, but no **2** was detected.

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