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Isolation and Structural Elucidation of the Degradation Products of Pregnanediol Disulfate obtained by Hot Acid Hydrolysis (Clinical Analysis on Steroids. XXI[1])

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Besides the main products, 17α-ethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**7a**), 17α-methyl-D-homo-5β-androstane-3α,17aβ-diol (**8**), and 17α-methyl-17aβ-chloro-D-homo-5β-androstan-3α-ol (**9**), other minor degradation products of pregnanediol 3,20-disulfate (**2**) were obtained under reflux in 3 N hydrochloric acid, and were structurally identified by comparison with synthetic specimens.

The steroidal mono olefins isolated were 5β-pregn-3-en-20α-ol (**10a**), 5β-pregn-2-en-20α-ol (**11a**), 5β-pregn-20-en-3α-ol (**12a**), and 5β-pregn-17(20)-en-3α-ol (**13a**). The *Z*-isomer of **13a**, 5β-pregn-17(20)-en-3α-ol (**14a**), was detected by gas chromatography and identified by gas chromatography–mass spectrometric comparison with synthetic compound.

Steroidal dienes were obtained as a mixture of Δ^2- and Δ^3-compounds having partial D-ring structures corresponding to **7a**, **12a**, and **13a**.

Although it was a minor product, 5β-pregnane-3α,20β-diol (**16**) was also obtained, and its isolation suggests the formation of a C_{20}-carbonium ion in the course of the hydrolysis.

The yield of intact steroid, pregnanediol (**1**), was only 18.4% of the total products obtained.

Keywords——pregnanediol; pregnanediol sulfates; hydrolysis; rearrangement reaction; D-homosteroid; NMR; gas chromatography

Introduction

Previously, we have reported the isolation of 17α-ethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (**7a**) as a urinary steroid,[2] and revealed that the compound was an artifact formed from pregnanediol 3,20-disulfate (**2**) by heating in 3 N hydrochloric acid.[3] Recently, we have isolated two D-homosteroids as the main products after **7a** from the same hydrolyzate of **2** and determined their structures as 17α-methyl-D-homo-5β-androstane-3α,17aβ-diol (**8**) and 17α-methyl-17aβ-chloro-D-homo-5β-androstan-3α-ol (**9**), respectively.[4]

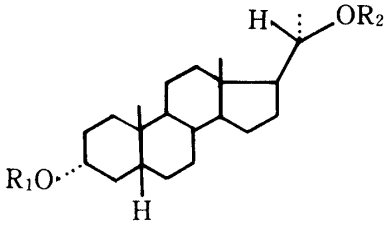
To elucidate the mechanism of formation of such rearranged steroids as **7a**, **8** and **9**, it became necessary to determine the structures of other minor degradation products formed concurrently in the hydrolysis. In this paper, we describe the isolation of these minor products and their structural elucidation based on the results obtained in preliminary experiments on 5α-pregnane-3β, 20α-diol derivatives.[5,6]

Results

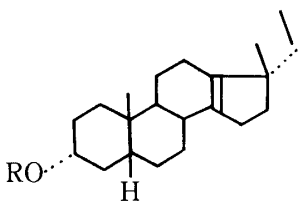
I) Isolation of the Hydrolyzates

Pregnanediol 3,20-disulfate (**2**, as dipotassium salt, 7.26 g)[7] was refluxed in 3 N hydrochloric acid for 30 min to give the hydrolyzate (3.60 g). The gas chromatogram of the hydrolyzate is shown in Fig. 1; it is clear that **7a** is a main hydrolysis product.

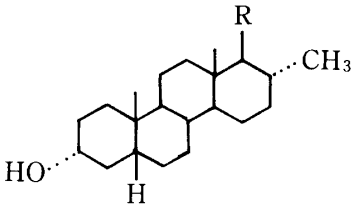
The degradation products were separated by repeating the column chromatographies as summarized in Fig. 2. The hydrolyzate was subjected to column chromatography (C-1) on



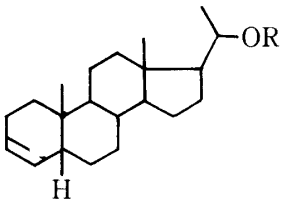
1 : $R_1=H$, $R_2=H$
2 : $R_1=SO_3K$, $R_2=SO_3K$
3 : $R_1=H$, $R_2=CH_3CO$
4 : $R_1=$tosyl, $R_2=CH_3CO$
5 : $R_1=CH_3CO$, $R_2=H$
6 : $R_1=CH_3CO$, $R_2=$tosyl



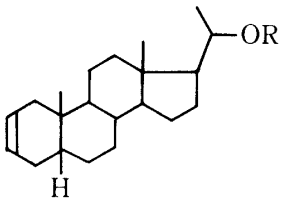
7a : $R=H$
7b : $R=CH_3CO$



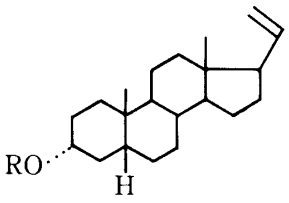
8 : $R=OH$
9 : $R=Cl$



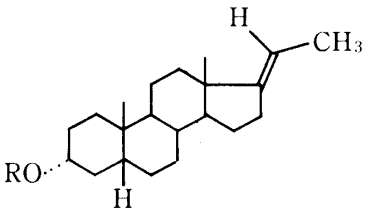
10a : $R=H$
10b : $R=CH_3CO$
10c : $R=SO_3K$



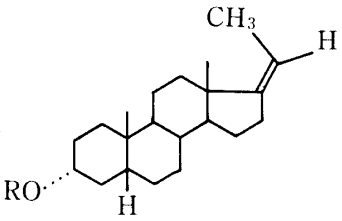
11a : $R=H$
11b : $R=CH_3CO$
11c : $R=SO_3K$



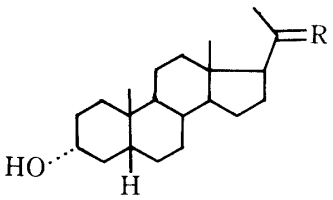
12a : $R=H$
12b : $R=CH_3CO$



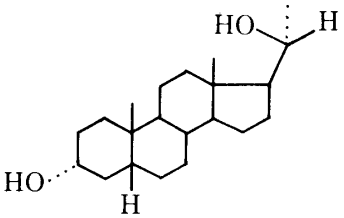
13a : $R=H$
13b : $R=CH_3CO$



14a : $R=H$
14b : $R=CH_3CO$



15a : $R=O$
15b : $R=N\text{-}NH\text{-}SO_2\text{-}C_6H_4\text{-}CH_3$



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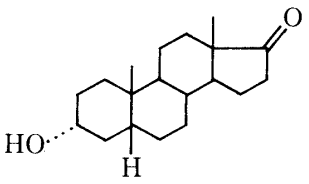

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tosyl : $SO_2\text{-}C_6H_4\text{-}CH_3$

Chart 1

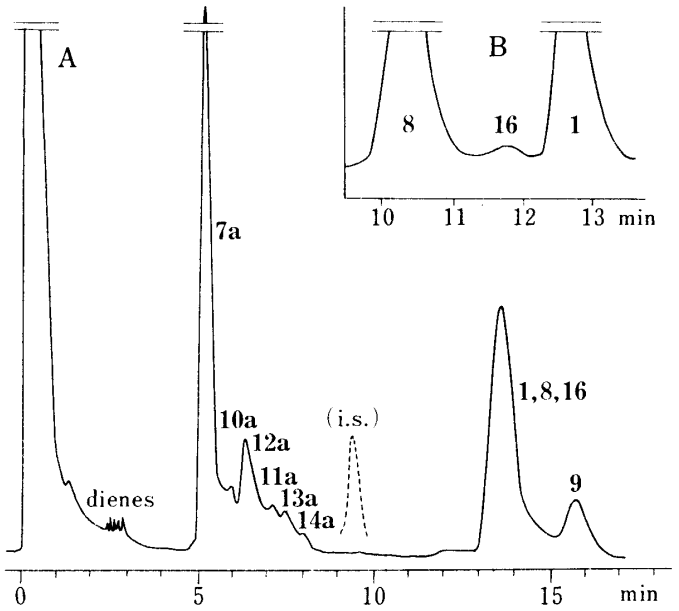


Fig. 1. Gas Chromatograms of the Hydrolyzate of Pregnanediol 3,20-Disulfate (A) and of Its Trimethylsilyl Ether (B)

i.s.: internal standard; estradiol 3-methyl ether. The chromatogram (B) shows only a part of the diol fraction.

silica gel, and the eluates were divided into the following four fractions: Fr. 1 (steroidal diene fraction), Fr. 2 (monohydroxy steroid fraction), and Fr. 3 and 4 (dihydroxy steroid fractions).

Recrystallization of Fr. 2 from *n*-hexane gave crystalline **7a** (Fr. 5). The mother liquor (Fr. 6) was again subjected to column chromatography (C-2) using silica gel impregnated with silver nitrate. By monitoring the components of each fraction by gas chromatography (GC), the eluates were divided into four fractions: Fr. 7, 8, 9, and 10.

Recrystallization of Fr. 7 from *n*-hexane gave further **7a** (Fr. 11) as a main product. Careful column chromatography (C-3) of the mother liquor (Fr. 12) using silica gel impregnated silver nitrate gave three new crystalline materials. Their structures were determined as 5β-pregn-3-en-20α-ol (**10a**), 5β-pregn-2-en-20α-ol (**11a**), and 5β-pregn-20-en-3α-ol (**12a**) by instrumental analyses and comparison with synthetic steroids.

A partial nuclear magnetic resonance (NMR) spectrum of **12a** is shown in Fig. 3 as an example. Signals at 5.76 ppm (octet) are attributable to the C_{20}-proton (Ha in Fig. 3), and signals at 4.96 and 4.95 ppm are assigned to the C_{21}-proton (Hb and Hc). Irradiation of the signals at 4.96 ppm changed the peaks at 5.76 ppm to a doublet (J=6.9 Hz). Conversely, irradiation at 5.76 ppm changed the signals at 4.95—4.96 ppm to a doublet (J=2.5 Hz). The spectral and splitting patterns of these olefinic protons, and also the spectral changes caused by decoupling, resemble those of a bile acid derivative having the same terminal ethylenic structure.[8]

Recrystallization of Fr. 8 from a mixture of *n*-hexane and benzene gave a chlorine-containing material (Fr. 13), which was identified as **9**.[4] By gas chromatographic analyses, the mother liquor (Fr. 14) was shown to contain a minor unknown material in addition to **7a**, **9**, **10a**, and **12a**. Because this unknown material (corresponding to **13a**) is the main component in Fr. 18, the separation will be discussed below.

Recrystallization of Fr. 9 from acetone gave **12a** (Fr. 15) and mother liquor (Fr. 16). Column chromatography (C-4) of Fr. 16 with silver nitrate-containing silica gel was again carried out and the eluates were divided into three fractions: Fr. 17, 18, and 19. Recrystallization of Fr. 18 gave a crystalline material (Fr. 20), which was assigned as 5β-pregn-17(20)-en-3α-ol (**13a**, *E*-isomer) and was identical with the synthetic specimen. By gas chromatographic

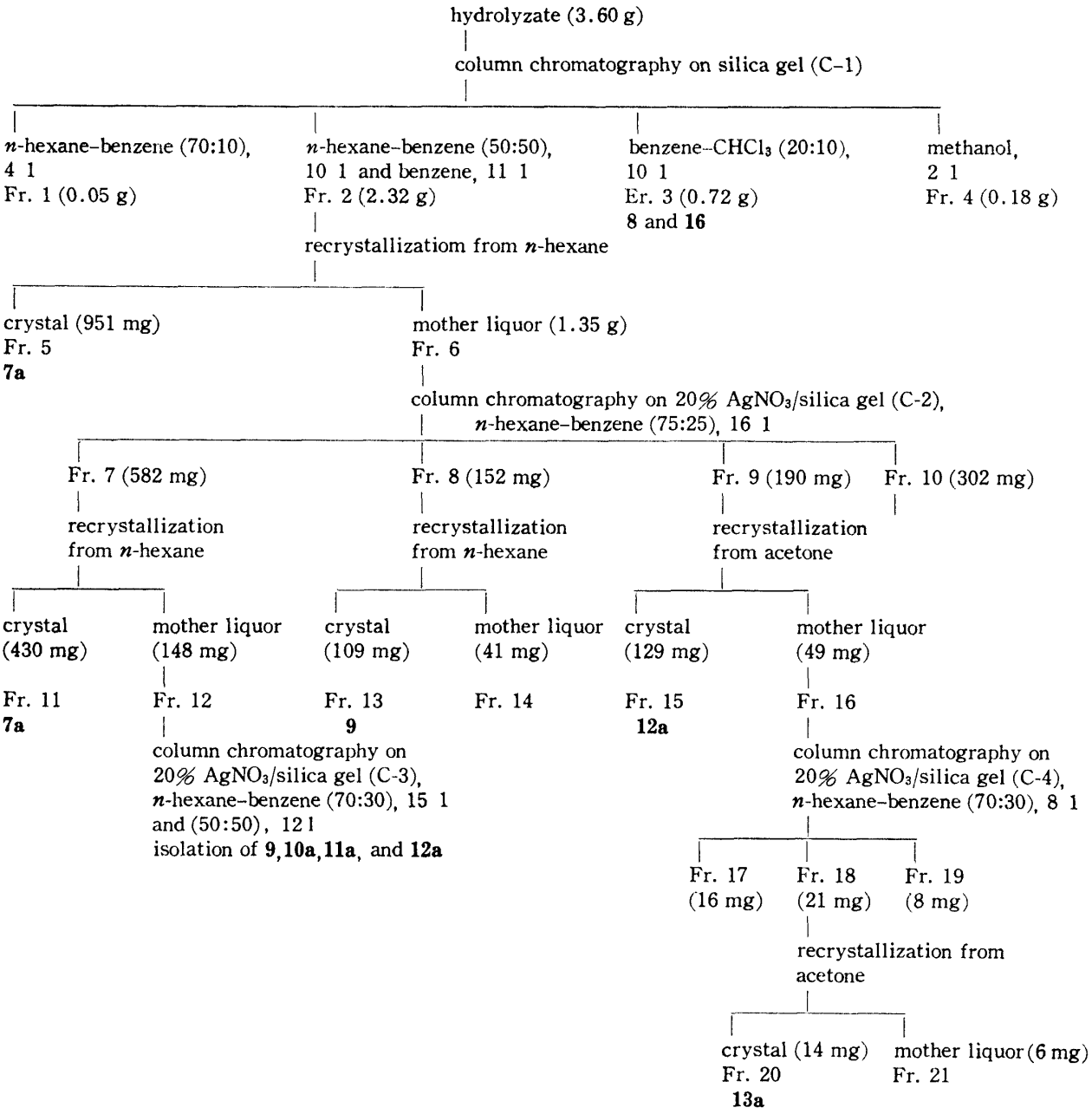


Fig. 2. Flow Sheet for the Separation of Degradation Products of Pregnanediol 3,20-Disulfate

The components of each fraction are shown in Table I.

analyses, Frs. 19 and 21 were shown to contain **9**, **10a**, **11a**, **12a**, and **13a**, and additional unknown material. This unknown material, however, could not be isolated because of its small amount. It was considered to be the *Z*-isomer of **13a** on the basis of the mass spectral fragmentation pattern in gas chromatography–mass spectrometry (GC–MS). Comparisons of the relative retention times of synthetic compound (**14a**) and its acetate (**14b**) with those of the corresponding peak of Fr. 19 and acetylated Fr. 19 are shown in Tables II and III.

Fr. 4 was subjected to preparative thin-layer chromatography (prep. TLC) and a mixture of **8** and 5β-pregnane-3α,20β-diol (**16**) was obtained. The complete separation of **8** and **16** was so difficult that they were separated as their dibenzoates again by prep. TLC. Saponification of the separated dibenzoate of **16** gave fine crystals of **16**, which were identical with authentic 5β-pregnane-3α,20β-diol. Fr. 3 and 10 were shown by GC to contain **1**, **8**, **9**, **16**, and other minor contents which were also detected in Fr. 4.

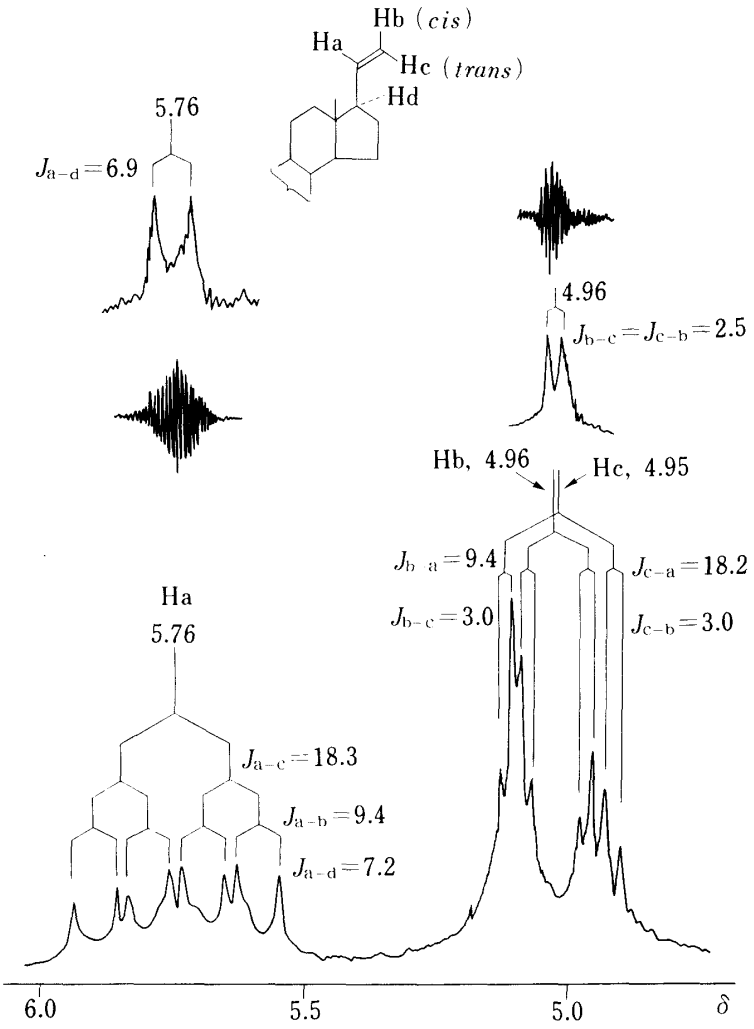


Fig. 3. A Partial Nuclear Magnetic Resonance Spectrum of **12a** at 100 M Hz in $CDCl_3$

The two top spectra show the results of spin decoupling experiments.

Lastly, experiments on Fr. 1 were carried out. The GC of this fraction has at least seven detectable peaks; GC–MS gave a molecular ion at *m/e* 284, corresponding to the molecular ion of steroidal diene (pregnanediol–$2\times H_2O$). Thus, these peaks are considered to be a mixture of steroidal dienes of Δ^2- or Δ^3-olefin type having such D-ring partial structures as **7a**, **12a**, **13a** (and **14a**). This was confirmed by the following experiments.

The olefinic steroids, **10a** and **11a**, were treated with sulfur trioxide-pyridine complex to give the corresponding sulfates, potassium 5β-pregn-3-en-20α-yl sulfate (**10c**) and potassium 5β-pregn-2-en-20α-yl sulfate (**11c**), respectively. Hot acid hydrolysis of **10c** and **11c** gave hydrolyzates, the gas chromatograms of which gave peaks coinciding with half of the peaks of Fr. 1.

II) Synthesis of Authentic Steroids

1) **Compounds 10a and 11a**——Treatment of 20α-acetoxy-5β-pregnan-3α-ol (**3**)[7] with *p*-toluenesulfonyl chloride gave 3α-tosyloxy-5β-pregnan-20α-yl acetate (**4**). The tosylate was refluxed in 2,6-lutidine as described by Blickenstaff and Foster[9] to give a mixed product, which was immediately saponified under alkaline conditions. The product obtained was subjected to column chromatography on silica gel containing silver nitrate, by which two crystalline pure materials were separated. On the basis of their NMR spectra, they were assigned as Δ^2- and Δ^3-steroid **11a** and **10a**, respectively.

TABLE I. The Yields of Degradation Products and Their Contents in Chromatographic Fractions[a]

Fr.	Weight (mg)	Diene fraction Dienes (%)	Monohydroxy steroid fractions (%)							Dihydroxy steroid fractions (%)			
			7a	**10a**	**11a**	**12a**	**13a**	**14a**	**9**	**1**	**8**	**16**	Others
1	50	98.0	1.8	0.2	—	—	—	—	—	—	—	—	—
3	720	—	—	—	—	—	—	—	7.6	64.3	25.3	1.7	1.0
4	180	—	—	—	—	—	—	—	—	50.3	28.2	1.1	20.4
5	951	—	100.0	—	—	—	—	—	—	—	—	—	—
10	302	—	—	—	—	—	—	—	44.5	11.1	41.4	1.0	2.0
11	430	—	100.0	—	—	—	—	—	—	—	—	—	—
12	148	—	33.8	25.7	13.2	27.2	—	—	—	—	—	—	—
13	109	—	—	—	—	—	—	—	100.0	—	—	—	—
14	41	—	0.7	3.6	2.1	30.2	5.4	—	58.0	—	—	—	—
15	129	—	—	—	—	100.0	—	—	—	—	—	—	—
17	16	—	—	2.0	3.0	24.0	70.0	—	1.0	—	—	—	—
19	8	—	—	—	—	32.0	52.0	2.0	14.0	—	—	—	—
20	14	—	—	—	—	—	100.0	—	—	—	—	—	—
21	6	—	—	3.0	—	45.3	43.0	2.0	7.0	—	—	—	—
Total	3130												
Yield, %		1.6	45.8	1.3	0.7	6.1	1.1	0.01	10.3	18.8	11.4	0.6	1.6

a) The yields of components of the fractions were determined by gas chromatography using the peak height method. The yields (%) are expressed as percentages of total weight (3130 mg) obtained.

TABLE II. Comparison of the Relative Retention Times of Monohydroxy Steroids obtained by the Hydrolysis of Pregnanediol 3,20-Disulfate with Those of Synthetic Specimens

Materials		Relative retention time[a]					
Hydrolyzates[b]		0.552	0.658	0.676	0.702	0.732	0.795
Synthetic	**7a**[c]	0.554	—	—	—	—	—
	10a	—	0.658	—	—	—	—
	11a	—	—	—	0.701	—	—
	12a	—	—	0.677	—	—	—
	13a	—	—	—	—	0.733	—
	14a	—	—	—	—	—	0.791

a) Internal standard (estradiol 3-methyl ether)=1.000 (11.95 min).
b) Relative retention times of hydrolyzates are all those of pure compounds isolated except for the material having a relative retention time of 0.795, which was detected in Frs. 19 and 21.
c) Reference 3.

TABLE III. Comparison of the Relative Retention Times of Monohydroxy Steroidal Acetates obtained by the Hydrolysis of Pregnanediol 3,20-Disulfate with Those of Synthetic Specimens

Materials		Relative retention time[a]					
Hydrolyzates[b]		1.446	1.814	1.894	1.954	2.009	2.208
Synthetic	**7b**[c]	1.447	—	—	—	—	—
	10b	—	1.814	—	—	—	—
	11b	—	—	—	1.955	—	—
	12b	—	—	1.893	—	—	—
	13b	—	—	—	—	2.010	—
	14b	—	—	—	—	—	2.210

a) Internal standard (**7a**)=1.000 (5.65 min).
b) Relative retention times of hydrolyzates are all those of pure acetates obtained except for the material having a relative retention time of 2.208, which was detected in acetylated Frs. 19 and 21.
c) Reference 3.

2) **Compound 12a**——This was obtained by the method of Schow and McMorris.[10] Treatment of 3α-hydroxy-5β-pregnan-20-one (**15a**) with *p*-toluenesulfonylhydrazide and hydrochloric acid in refluxing ethanol afforded the unstable tosylhydrazone (**15b**). The tosylhydrazone with butyl lithium in dry ether gave the desired **12a**.

3) **Compound 13a**——Monoacetate, 3α-acetoxy-5β-pregnan-20α-ol (**5**)[7] was derivatized to its corresponding tosylate, 20α-tosyloxy-5β-pregnan-3α-yl acetate (**6**) under the conditions described. Refluxing of **6** in pyridine according to Lebceuf *et al.*[11] gave 3α-acetoxy-5β-pregn-17(20)-ene (**13b**). Saponification of **13b** under alkaline conditions gave **13a** quantitatively.

4) **Compound 14a**——According to the method of Krubinor and Oliveto,[12] the Wittig reaction of 3α-hydroxy-5β-androstan-17-one (ethiocholanolone, **17**) using ethyl bromide and sodium hydride in dry dimethyl sulfoxide followed by repeated crystallization of the product gave the desired olefin **14a**.

In Tables II and III, the relative retention times of monohydroxy steroidal olefins which were isolated as hydrolysis products of **2** and their acetates were compared with those of the synthetic steroids. Complete identity was observed between the isolated and the synthetic products.

Discussion

Table I shows the yields of the degradation products obtained by the hot hydrochloric acid hydrolysis of pregnanediol disulfate (**2**), from which it can be seen that **7a** accounts for 46.7% of the total products. This is about twice as much as that of Δ^{13}-steroid obtained from 5α-pregnane-3β,20α-diol disulfate.[13] The difference between these yields means that the formation of Δ^{13}-steroid from 20α-ol sulfate is affected by the A/B-ring junction of the steroid molecule.

Two D-homosteroids, **8** and **9**, were obtained as the next most abundant products, which means that D-homoannulation is never a minor reaction in the hydrolysis of **2**, although it occurs specifically in the 20β-ol sulfate.[14]

The yield of olefinic isomers at the side chain are; 6 percent for **12a**, 1.5 percent for **13a**, and a negligible amount for **14a**. This means that the elimination reaction occurred mainly at the C_{20-21} bond.

The elimination reaction at the A-ring amounts to about 2 percent. This means that elimination of the equatorial hydroxyl group is difficult, even if it is esterified with sulfuric acid. Further elimination of these olefins, **10a**, and **11a**, to dienes might be difficult, since the total yield of diene fraction (Fr. 1) was only 1.5 percent. This may be due to their insolubilities in aqueous solvent, because water-soluble sulfates (**10c** and **11c**) on hydrolysis gave a mixture of dienes in high yield.

The intact steroid, pregnanediol (**1**), was obtained in only 18 percent yield, which means that a significant amount of **1** was destroyed in the course of hydrolysis. This is in accord with the result by Metcalf,[15] who investigated kinetically the degradation of conjugated pregnanediol by hydrochloric acid at elevated temperature.

It is worth noting that 5β-pregnane-3α,20β-diol (**16**) was obtained, though in a yield of only 0.6 percent. The isolation of this 20β-ol suggests that a C_{20}-carbonium ion might be formed by cleavage of the carbon–oxygen bond of the 20α-sulfooxy group during the hydrolysis. Similar isomerization of a steroidal alcohol during hydrolysis was observed in the case of androsterone sulfate.[16]

In the previous paper,[2] we speculated that a concerted mechanism is involved in the rearrangement reaction of **2** to **7a**. From the present finding of formation of the 20β-ol (**16**), however, it is possible that a stepwise mechanism is also participating in the formation of **7a**. Further, a C_{20}-carbonium ion is thought to be a precursor of such mono olefins as **12a**, **13a**, and **14a** as well as **7a**. This is also the case for the formation of two D-homosteroids, **8** and **9**.[4]

Details of the mechanism(s) of these rearrangement reactions will be presented in forthcoming papers.

Experimental

Melting points were determined on a Kofler-type micro-hot stage (Mitamura, Tokyo) and are uncorrected. TLC was performed with Merck precoated silica gel 60 F_{254} plates. Preparative TLC was done with the same commercial product, 20×20 cm, with a thickness of 0.5 mm. NMR spectra were measured on a JNM FX-100 spectrometer (JEOL, Tokyo) at 100 MHz and chemical shifts are expressed relative to 1% tetramethylsilane as an internal standard. *J*-Values of fine structures were obtained by expanding the abscissa 4—10 times, and were read to the order of 0.1 Hz with an error of ±0.24 Hz. Symbols, s, singlet; d, doublet; t, triplet; q, quartet; sept, septet; oct, octet; m, multiplet; q-t, quartet-triplet; d-t, doublet-triplet; br s, broad singlet; tr, *trans*; c, *cis*. Infrared spectra (IR, ν_{max}) in KBr disks were recorded on a JASCO IR-2 machine (Nihon Bunko, Tokyo) and are expressed as cm^{-1}. MS were taken by the direct insertion method with a 9000 B machine (Shimadzu, Kyoto). GC was carried out on a 4 CM gas chromatograph (Shimadzu, Kyoto) using a glass column (2 m×3 mm, i.d.) packed with 1.5% OV-1 on Shimalite W (80—100 mesh) with nitrogen as a carrier gas at the flow rate of 30 ml/min. The column temperatures employed were 210 and 230°C for the analyses of free and acetylated steroids, respectively. As internal standards, estradiol 3-methyl ether for analyses of free steroids, and **7a** for analyses of steroidal acetates, were used. Trimethylsilylation was done by using TMSI-H (a pyridine solution of hexamethyldisilazane and trimethylchlorosilane, Gaskuro Kogyo Co., Ltd., Tokyo). The trimethylsilyl (TMS) ethers were analyzed at a column temperature of 230°C under the same conditions as described above. GC–MS analyses were done by combination of the above mass spectrometer with a 4 CM gas chromatograph with the same column as described above and with helium as a carrier gas at the flow rate of 25 ml/min. Other conditions employed were as follows; column temperature 270°C, ionizing voltage 70 eV. Elemental analyses were done by the staff of the Analytical Center of Hokkaido University (Sapporo), to whom our thanks are due. Steroidal materials, 5β-pregnane-3α,20α-diol, 5β-pregnane-3α,20β-diol, and 3α-hydroxy-5β-androstan-17-one (ethiocholanolone), were obtained from Steraloids (N.H., USA). 3α-Hydroxy-5β-pregnan-20-one was obtained from Sigma (St. Louis, USA). Estradiol 3-methyl ether was obtained in this laboratory by treatment of estradiol (Teikoku Hormone, Tokyo) with diazomethane. Silica gel used for column chromatography was Merck Kieselgel 60 (60—230 mesh). All the chemicals used were of reagent grade, and were used without further purification.

I) Isolation

Hydrolysis of 2 and Column Chromatography (C-1) of the Hydrolyzate——Boiling 6 N HCl (300 ml) was added to refluxing aqueous solution (300 ml) of **2** (7.26g),[7] and the mixture was refluxed. After 30 min, the solution was cooled to room temperature. The oily precipitate formed was removed and the aqueous solution was extracted with ether. The extract and the precipitate were combined, washed with water, dried over anhydrous Na_2SO_4, and concentrated. The oily residue (3.60 g) was chromatographed on silica gel (150 g, i.d., 3 cm) and eluted with organic solvent as shown in Fig. 2. Fractions (50 ml) were collected automatically, monitored by TLC and combined accordingly. The combined eluates were divided into four fractions (Fr. 1, 2, 3, and 4) according to their components.

17α-Ethyl-17β-methyl-18-nor-5β-androst-13-en-3α-ol (7a)——Recrystallization of Fr. 2 gave a crystalline material (Fr. 5, 951 mg), which was identified as **7a**, mp 201—202°C (200—202°C).[2,3] The acetate 17α-ethyl-17β-methyl-18-nor-5β-androst-13-en-3α-yl acetate (**7b**) recrystallized from methanol: mp 168—170°C (169—171°C).[2,3] The mother liquor (Fr. 6) was used for the following experiments.

Column Chromatography (C-2) of Fr. 6——Fr. 6 (1.35 g) was applied to a column (i.d., 2 cm) packed with 20% $AgNO_3$–silica gel (100 g) and eluted with 16 l of a mixture of *n*-hexane and benzene (75: 25). Fractions (50 ml) were collected automatically and monitored by GC. Eluates were divided into four fractions, Fr. 7, 8, 9, and 10, as shown in Fig. 2. Recrystallization of Fr. 7 from *n*-hexane gave further **7a** (430 mg, Fr. 11). The mother liquor (148 mg, Fr. 12) was subjected to the following column chromatography.

Column Chromatography (C-3) of Fr. 12——Fr. 12 (147 mg) was chromatographed on a column (i.d., 1 cm) packed with 20% $AgNO_3$–silica gel (50 g) and eluted with 15 l of a mixture of *n*-hexane and benzene (70: 30). Two crystalline materials were separated as pure compounds and identified as **7a** (34 mg) and **10a** (20 mg). Further elution with 12 l of a mixture of *n*-hexane and benzene (50: 50) gave another two pure materials, **11a** (8 mg) and **12a** (22 mg). Besides these pure materials, an oily mixture (51 mg) of the above compounds was obtained. The chemical properties of these compounds are summarized below, 1)—6)

1) **5β-Pregn-3-en-20α-ol (10a)**——Crystallization from *n*-hexane gave fine needles, mp 122.5—123°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48); C, 83.38; H, 11.33. Found: C, 83.49; H, 11.26. IR: 3350, 3025, 2950—2850, 1660. NMR ($CDCl_3$) δ: 5.68—5.52 (1H, m, C_3–H), 5.35 (1H, q, J_1=10.3 Hz, J_2=1.7 Hz, C_4–H), 3.69 (1H, oct, J_{20-17}=8.0 Hz, J_{20-21}=6.0 Hz, C_{20}–H), 1.21 (3H, d, J=6.3 Hz, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.66 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$]. 257. No depression of melting point was observed on admixture of this material with authentic steroid. The spectral

properties were identical with those of the synthetic specimen.

2) **20α-Acetoxy-5β-pregn-3-ene (10b)**——Five mg of **10a** was acetylated in the usual manner with acetic anhydride in pyridine to give a crude acetate (5.3 mg), which was recrystallized from methanol to afford plates, mp 119.5—120°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.30; H, 10.36. IR: 3025, 2950—2850, 1735. NMR ($CDCl_3$) δ: 5.76—5.56 (1H, m, C_3-H), 5.30 (1H, q, $J_1=10.6$ Hz, $J_2=1.8$ Hz, C_4-H), 4.91 (1H, oct, $J_{20-17}=8.0$ Hz, $J_{20-21}=6.1$ Hz, C_{20}-H), 2.00 (3H, s, CH_3COO), 1.20 (3H, d, $J=6.1$ Hz, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3). MS *m/e*: 344 (M^+), 284 (M^+-HOAc), 269 [$M^+-(CH_3+HOAc)$]. No depression of melting point was observed on admixture of the material with an authentic sample.

3) **5β-Pregn-2-en-20α-ol (11a)**——The material was recrystallized from *n*-hexane to give fine needles, mp 123°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.51; H, 11.40. IR: 3350—3300, 3030, 2950—2850, 1645. NMR ($CDCl_3$) δ: 5.60—5.45 (2H, m, C_2- and C_3-H), 3.69 (1H, oct, $J_{20-17}=8.1$ Hz, $J_{20-21}=6.1$ Hz, C_{20}-H), 1.20 (3H, d, $J=6.1$ Hz, 21-CH_3), 0.97 (3H, s, 19-CH_3), 0.65 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$]. No depression of melting point was observed on admixture of this compound with an authentic sample.

4) **20α-Acetoxy-5β-pregn-2-ene (11b)**——Acetylation of **11a** (3 mg) in the usual way gave 3.2 mg of crude acetate, which was recrystallized from methanol to afford fine needles, mp 124—125°C. IR: 3030, 2940—2850, 1737, 1660. NMR ($CDCl_3$) δ: 5.65—5.40 (2H, m, C_2- and C_3-H), 4.91 (1H, cct, $J_{20-17}=7.9$ Hz, $J_{20-21}=6.1$ Hz, C_{20}-H), 2.00 (3H, s, CH_3COO), 1.20 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3). MS *m/e*: 344 (M^+), 284 (M^+-HOAc), 269 [$M^+-(CH_3+HOAc)$]. No depression of mixed melting point was observed on admixture of this material with authentic steroid.

5) **5β-Pregn-20-en-3α-ol (12a)**——The pure material was recrystallized from acetone to give fine needles, mp 153—155°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.29; H, 11.26. IR: 3250, 2910, 2850, 1630, 1460, 1445. NMR ($CDCl_3$) δ: 5.76 (1H, oct, $J_{20-21(tr)}=18.3$ Hz, $J_{20(c)-21}=9.4$ Hz, $J_{20-17}=7.2$ Hz, C_{20}-H), 4.96 (1H, q, $J_{21-21(c)}=9.4$ Hz, $J_{21-21}=3.0$ Hz, $C_{21(c)}$-H), 4.95 (1H, q, $J_{21(tr)-20}=18.2$ Hz, $J_{21-21}=3.0$ Hz, $C_{21(tr)}$-H), 3.62 (1H, sept, $J_1=J_{1'}=10.2$ Hz, $J_2=J_{2'}=5.0$ Hz, 3β-H), 0.92 (3H, s, 19-CH_3), 0.56 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$]. No depression of mixed melting point was observed on admixture of this steroid with an authentic sample.

6) **3α-Acetoxy-5β-pregn-20-ene (12b)**——Eight mg of **12a** was acetylated in the usual manner to afford 9 mg of crude acetate, recrystallization of which from methanol gave fine needles, mp 120.5—123°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.25; H, 10.58. IR: 3080, 2950, 2870, 1735, 1630, 1470, 1450. NMR ($CDCl_3$) δ: 5.71 (1H, oct, $J_{20-21(tr)}=18.0$ Hz, $J_{20-21(c)}=9.0$ Hz, $J_{20-17}=7.0$ Hz, C_{20}-H), 5.04—4.60 (3H, m, C_{21}- and 3β-H), 2.03 (3H, s, CH_3COO), 0.94 (3H, s, 19-CH_3), 0.57 (3H, s, 18-CH_3). MS *m/e*: 344 (M^+), 284 (M^+-HOAc), 269 [$M^+-(CH_3+HOAc)$]. No depression of melting point was observed on admixture of the material with synthetic steroid.

17α-Methyl-17aβ-chloro-D-homo-5β-androstan-3α-ol (9)——Recrystallization of Fr. 8 (152 mg) from a mixture of *n*-hexane and benzene gave fine needles (109 mg), mp 208—209°C (207—209°C),[4] which were identified as **9** by comparison with an authentic specimen. The mother liquor (41 mg, Fr. 14) was shown by gas chromatography to contain **7a**, **9**, **10a**, **12a**, and a very small amount of another material (corresponding to **13a**), on which the experiments were carried out (Fr. 21).

Treatment of Fr. 9——Recrystallization of Fr. 9 (190 mg) from acetone gave fine needles, mp 153—154°C, which were identified as **12a**. The mother liquor (49 mg, Fr. 16) was used for the following experiment.

Column Chromatography (C-4) of Fr. 16——Fr. 16 (48 mg) was applied to a column (i.d., 0.5 cm) of 20% $AgNO_3$–silica gel (10 g) and eluted with 8 l of a mixture of *n*-hexane and benzene (70: 30). On the basis of gas chromatography of each fraction (50 ml), the eluates were divided into the following three fractions: Fr. 17 (16 mg), Fr. 18 (21 mg), and Fr. 19 (8 mg).

5β-Pregn-17(20)-en-3α-ol (13a)——Recrystallization of Fr. 18 (21 mg) from *n*-hexane gave fine needles (14 mg, Fr. 20), mp 128—130°C. The mother liquor (Fr. 21) was used for gas chromatographic analysis. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.36; H, 11.30. IR: 3250, 2975, 2930, 1470. NMR ($CDCl_3$) δ: 5.20 (1H, q-t, $J_{20-21}=7.0$ Hz, $J_{20-16}=2.5$ Hz, C_{20}-H), 3.63 (1H, sept, $J_1=J_{1'}=10.0$ Hz, $J_2=J_{2'}=5.0$ Hz, 3β-H), 2.35—2.00 (2H, br s C_{16}-H), 1.53 (3H, d-t, $J_{21-20}=7.0$ Hz, $J_{21-16}=1.5$ Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.73 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$]. No depression of melting point was observed on admixture of the material with authentic steroid.

3α-Acetoxy-5β-pregn-17(20)-ene (13b)——Acetylation of **13a** (7 mg) by the usual procedure gave the crude acetate (7.3 mg), which was recrystallized from a mixture of *n*-hexane and ether. Fine needles were obtained, mp 119—121°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 79.98; H, 10.76. IR: 2925, 2850, 1730, 1445, 1360, 1255. NMR ($CDCl_3$) δ: 5.02 (1H, q-t, $J_{20-21}=7.0$ Hz, $J_{20-16}=2.4$ Hz, C_{20}-H), 4.72 (1H, sept, $J_1=J_{1'}=10.8$ Hz, $J_2=J_{2'}=5.4$ Hz, 3β-H), 2.35—2.10 (2H, br s, C_{16}-H), 2.02 (3H, s, CH_3COO), 1.53 (3H, d-t, $J_{21-20}=7.0$ Hz, $J_{21-16}=1.5$ Hz, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.72 (3H, s, 18-CH_3). MS *m/e*: 344 (M^+), 284 (M^+-HOAc). No depression of melting point was observed on admixture of the acetate with synthetic steroid.

Identification of 14a in Fr. 19 and Fr. 21——By gas chromatographic analyses, Fr. 17 (16 mg), Fr. 19

(8 mg), and Fr. 21 (6 mg), were shown to contain **9**, **10a**, **11a**, **12a**, and **13a**. Additional minor unknown material (corresponding to **14a**) was detected in Frs. 19 and 21. This unknown material had a relative retention time (rt_R) of 0.759, which was the same as that of synthetic **14a** (Table II). The MS (GC-MS) of this material, *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$], was identical with that of synthetic **14a**. The rt_R of this acetate (acetylated Fr. 19 and Fr. 20) and that of synthetic acetate are compared in Table III.

5β-Pregnane-3α,20β-diol (16)——Preparative TLC of 300 mg of Fr. 3 was carried out using a mixture of *n*-hexane–acetone–chloroform–methanol (80: 20: 6: 3) in multiple runs. Because the complete separation of **8** and **16** was impossible, the band corresponding to **16** (and also to **8**) was scraped off and extracted with ethyl acetate. The mixture (13 mg) obtained was treated with benzoyl chloride in pyridine to give a mixture of benzoates (18 mg), which was again subjected to prep. TLC using a mixture of benzene and acetone (100: 1) in multiple runs. The band corresponding to 5β-pregnane-3α,20β-diol dibenzoate was scraped off and extracted with chloroform to give an oily material (5.4 mg), the homogeneity of which was indicated by NMR. For confirmation, the NMR spectrum of this material was compared with spectra of authentic dibenzoates of **1**, **8**, and **16**: 3α,20α-dibenzoyloxy-5β-pregnane, 3α,17aβ-dibenzoyloxy-D-homo-5β-androstane, and 3α,20β-dibenzoyloxy-5β-pregnane, respectively, in $CDCl_3$.

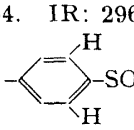
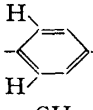
Dibenzoate of[a]	Aromatic (10H, m)	19-CH_3 (3H, s)	18-CH_3 (3H, s)	Others
1	8.20—7.30	0.97	0.77	5.30—4.70 (2H, m, 3β- and 20β-H)
8	8.20—7.30	1.00	0.93	5.20—4.70 (1H, m, 3β-H) and 4.60 (1H, J=9.6 Hz, 17aα-H)
16	8.25—7.30	0.90	0.67	5.20—4.75 (2H, m, 3β- and 20α-H)
Hydrolyzate	8.20—7.30	0.92	0.66	5.20—4.70 (2H, m, 3β- and 20α-H)

a) Synthesized in this laboratory.

Saponification of the benzoate in 1 N KOH in 90% methanol gave a crude free steroid (3.2 mg), recrystallization of which from aq. ethanol afforded fine needles, mp 231—234°C (231°C).[17] No depression of melting point was observed on admixture of the material with authentic steroid. MS *m/e*: 320 (M^+), 302 (M^+-H_2O), 284 ($M^+-2\times H_2O$).

Gas Chromatographic Identification of 16——To a pyridine solution (0.5 ml) of 0.4 mg of **16** and 0.2 mg of estradiol 3-methyl ether (internal standard), 0.2 ml of TMSI-H was added. The mixture was allowed to stand for 5 min with occasional shaking at room temperature. The supernatant was injected into the gas chromatograph. For comparison, the same procedure was carried out with authentic **1**, **8**, and **16**. Relative retention times of TMS ethers of authentic steroids, **1**, **8**, and **16**, were 1.820, 1.544, and 1.673, respectively. The TMS ether of the above material had rt_R 1.674 (TMS ether of estradiol 3-methyl ether=1.000, 5.10 min).

II) Synthetic Procedures

3α-Tosyloxy-5β-pregnan-20α-yl Acetate (4)——*p*-Toluenesulfonyl chloride (500 mg) was added to a solution of pregnanediol 20-monoacetate (**3**, 730 mg)[7] in pyridine (50 ml) and the solution was allowed to stand overnight at room temperature. Pyridine was removed under reduced pressure at 50°C and the resultant residue was dissolved in 20 ml of aq. acetone. The solution was stirred overnight at room temperature, and extracted with ether. The extract was washed with 5% $NaHCO_3$, 1 N HCl, water, and dried. Removal of the solvent gave a powder (1.12 g), which was recrystallized from ether, mp 152.5—153°C. *Anal.* Calcd for $C_{30}H_{44}O_5S$ (516.72): C, 69.73; H, 8.58; S, 6.21. Found: C, 69.98; H, 8.46; S, 6.54. IR: 2960—2940, 2880, 1735, 1600, 1380, 1360, 1260, 1195. NMR ($CDCl_3$) δ: 7.80 (2H, d, J=8.0 Hz, ), 7.33 (2H, d, J=8.0 Hz, ), 4.90 (1H, m, 20β-H), 4.45 (1H, sept, $J_1=J_{1'}=11.0$ Hz, $J_2=J_{2'}=5.5$ Hz, 3β-H), 2.45 (3H, s, CH_3–Ph), 2.01 (3H, s, CH_3COO), 1.21 (3H, d, J=6.0 Hz, 21-CH_3), 0.88 (3H, s, 19-CH_3), 0.64 (3H, s, 18-CH_3).

Solvolysis of 4 and Saponification——A solution of **4** (650 mg) in γ-collidine (20 ml) was refluxed for 2 h, then cooled to room temperature. The solution was diluted with 100 ml of ether, washed with 1 N HCl and water, then dried. Removal of the solvent gave 435 mg of oily product, which showed two peaks on GLC (rt_R: 1.814 and 1.955, the ratio of the peak height; about 2: 1, internal standard=1.000, 5.65 min). The mixture (430 mg) was dissolved in 10 ml of 1 N KOH in 95% aq. alcohol and allowed to stand overnight at room temperature. After removal of the solvent, the residue obtained was dissolved in ether. The solution was washed with water, dried, and concentrated. The product (410 mg) showed two peaks on GLC,

the rt_R values of which were 0.658 and 0.704 with peak areas of about 63% and 37%, respectively (internal standard, 11.55 min). The mixture was used for the following experiments.

Chromatographic Separation of Δ^2- and Δ^3-Steroids——The above mixture (410 mg) was applied to a column (i.d., 1.5 cm) packed with 20% $AgNO_3$–silica gel (150 g) and eluted with a mixture of *n*-hexane and benzene. By monitoring each fraction (50 ml) by GLC, the eluates were divided into four fractions (Fr. a—d).

Fr.	Solvent	Vol (l)	Weight (mg)	rt_R[a]
a	*n*-Hexane/Benzene (70: 30)	6.2	12	(impurity)
b	*n*-Hexane/Benzene (70: 30)	3.2	144	0.658
c	*n*-Hexane/Benzene (70: 30)	4.4	211	0.658 0.704
d	*n*-Hexane/Benzene (50: 50)	3.2	50	0.704

a) Estradiol 3-methyl ether = 1.000 (11.80 min).

1) **5β-Pregn-3-en-20α-ol (10a)**——The pure material from Fr. b was recrystallized from *n*-hexane to give compound **10a** as fine needles, mp 122.5—123°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.44; H, 11.33. IR: 3350—3300, 3025, 2950—2850, 1650, 1450—1440, 1370. NMR ($CDCl_3$) δ: 5.70—5.50 (1H, m, C_3–H), 5.36 (1H, q, J_1=10.2 Hz, J_2=1.8 Hz, C_4–H), 3.71 (1H, oct, J_{20-17}=8.0 Hz, J_{20-21}=6.1 Hz, C_{20}–H), 1.20 (3H, d, J=6.2 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$].

2) **20α-Acetoxy-5β-pregn-3-ene (10b)**——Compound **10a** (44 mg) was acetylated in the usual way to give crude acetate (44 mg), which was recrystallized from methanol to give compound **10b**, mp 119.5—120°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.24; H, 10.60. IR: 3025, 2950—2850, 1735, 1450, 1440, 1370, 1245. NMR ($CDCl_3$) δ: 5.72—5.52 (1H, m, C_2–H), 5.30 (1H, q, J_1=10.5 Hz, J_2=1.7 Hz, C_3–H), 4.90 (1H, oct, J_{20-17}=8.0 Hz, J_{20-21}=6.0 Hz, C_{20}–H), 2.02 (3H, s, CH_3COO), 1.21 (3H, d, J=6.1 Hz, 21-CH_3), 0.95 (3H, s, 19-CH_3), 0.66 (3H, s, 18-CH_3).

3) **5β-Pregn-2-en-20α-ol (11a)**——Fr. d was recrystallized from *n*-hexane to give compound **11a** as fine needles, mp 123°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.36; H, 11.42. IR: 3250, 3030, 2950—2850, 1660, 1445, 1375. NMR ($CDCl_3$) δ: 5.60—5.40 (2H, m, C_2– and C_3–H), 3.70 (1H, oct, J_{20-17}=8.1 Hz, J_{20-21}=6.0 Hz, C_{20}–H), 1.22 (3H, d, J=6.1 Hz, 21-CH_3), 0.98 (3H, s, 19-CH_3), 0.66 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$].

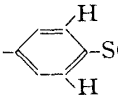
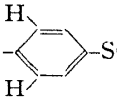
4) **20α-Acetoxy-5β-pregn-2-ene (11b)**——Acetylation of the above **11a** (22 mg) in the usual way gave the corresponding acetate **11b** (24 mg), which was recrystallized from methanol, mp 126.5°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.40; H, 10.68. IR: 3030, 2950—2850, 1735, 1640, 1450—1435, 1240. NMR ($CDCl_3$) δ: 5.65—5.50 (2H, m, C_2– and C_3–H), 4.90 (1H, oct, J_{20-17}=8.0 Hz, J_{20-21}=6.2 Hz, C_{20}–H), 2.01 (3H, s, CH_3COO), 1.21 (3H, d, J=6.1 Hz, 21-CH_3), 0.98 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3).

5β-Pregn-20-en-3α-ol (12a)——A solution containing 3α-hydroxy-5β-pregnan-20-one (**15a**, 460 mg), *p*-toluenesulfonylhydrazide (480 mg), and conc. HCl (0.1 ml) in absolute ethanol (20 ml) was refluxed for 2 h. The cooled reaction mixture was diluted with water (50 ml) and extracted with ether. The combined extract was washed with 5% HCl, water, and dried. Removal of the solvent yielded 680 mg of tosylhydrazone (**15b**). NMR ($CDCl_3$) δ: 7.86—7.30 (4H, m, aromatic), 3.54 (1H, m, 3β-H), 2.43 (3H, s, Ph–CH_3), 2.10 (3H, s, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.23 (3H, s, 18-CH_3). Although the product was shown to contain about 10% of the starting material by NMR, it was immediately used in the following experiment because of its instability.

A solution of butyllithium (7.5 ml of 1.6 M in *n*-hexane) in ether (15 ml) was added dropwise to a stirred solution of **15b** (650 mg) in dry ether (10 ml) under nitrogen at 0°C over a period of 10 min. After addition was complete, the mixture was allowed to stand at room temperature and stirred under nitrogen overnight. Water (5 ml) was slowly added and the aqueous solution was extracted with ether. The extract was washed with water, dried, and concentrated to give an oil (401 mg). Prep. TLC of the product using a mixture of cyclohexane and acetone (3: 1) gave a crystalline material **12a** (174 mg), recrystallization of which from acetone gave fine needles, mp 150—151°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.44; H, 11.21. IR: 3400, 2925, 2850, 1630. NMR ($CDCl_3$) δ: 5.76 (1H, oct, $J_{20-21(tr)}$=18.0 Hz, $J_{20-21(c)}$=9.0 Hz, J_{20-17}=7.0 Hz, C_{20}–H), 4.95 (1H, q, $J_{21(c)-20}$=9.3 Hz, J_{21-21}=3.1 Hz, $C_{21(c)}$–H), 4.95 (1H, q, $J_{21(tr)-20}$=18.1 Hz, J_{21-21}=3.1 Hz, $C_{21(tr)}$–H), 3.64 (1H, sept, $J_1=J_{1'}$=10.0 Hz, $J_2=J_{2'}$=5.0 Hz, 3β-H), 0.93 (1H, s, 19-CH_3), 0.57 (1H, s, 18-CH_3). MS *m/e*: 302 (M^+), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$].

3α-Acetoxy-5β-pregn-20-ene (12b)——The above compound (56 mg) was acetylated in the usual way to give the crude acetate **12b** (60 mg), recrystallization of which from methanol gave fine needles, mp 121—123°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.40; H, 10.67. IR: 2925, 2875, 1730, 1630, 1240. NMR ($CDCl_3$) δ: 5.74 (1H, oct, $J_{20-21(tr)}$=18.0 Hz, $J_{20-21(c)}$=9.0 Hz, J_{20-17}=7.0 Hz,

C_{20}–H), 5.03—4.57 (3H, m, 3β- and C_{21}–H), 2.03 (3H, s, CH_3COO), 0.93 (3H, s, 19-CH_3), 0.57 (3H, s, 18-CH_3). MS *m/e*: 344 (M^+), 284 (M^+-HOAc), 269 [$M^+-(CH_3+HOAc)$].

20α-Tosyloxy-5β-pregnan-3α-yl Acetate (6)——*p*-Toluenesulfonyl chloride (760 mg) was added to a pyridine solution (50 ml) of pregnanediol 3-monoacetate (**5**, 292 mg),[7] and the mixture was allowed to stand for 3 h at room temperature. After removal of the solvent under reduced pressure, the oily material obtained was dissolved in benzene. The solution was washed with 5% $NaHCO_3$, water, dried, and concentrated. The product (320 mg) was recrystallized from a mixture of ether and *n*-hexane to give compound **6** as fine needles, mp 124—126°C. *Anal.* Calcd for $C_{30}H_{44}O_5S$ (516.72): C, 69.73; H, 8.58; S, 6.21. Found: C, 70.01; H, 8.41; S, 6.52. IR: 2950—2850, 1724, 1595, 1350, 1165. NMR ($CDCl_3$) δ: 7.78 (2H, d, J=8.0 Hz, ), 7.30 (2H, d, J=8.0 Hz, ), 4.90—4.40 (2H, m, 3β- and 20β-H), 2.45 (3H, s, CH_3–Ph), 2.02 (3H, s, CH_3COO), 1.33 (3H, d, J=6.0 Hz, 21–CH_3), 0.92 (3H, s, 19-CH_3), 0.63 (3H, s, 18-CH_3).

3α-Acetoxy-5β-pregn-17(20)-ene (13b)——A pyridine solution (50 ml) containing 429 mg of **6** was refluxed for 1 h by the reported method[10] and cooled to room temperature. After removal of the solvent under reduced pressure, the oily product was dissolved in benzene (100 ml), and the solution was washed with 1 N HCl, 5% $NaHCO_3$ and water, then dried. Evaporation of the solvent gave a product **13b** (302 mg), which was recrystallized from a mixture of ether and *n*-hexane to give fine needles, mp 121—124°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 79.98; H, 10.64. IR: 2925, 2850, 1730, 1445, 1360, 1225. NMR ($CDCl_3$) δ: 5.02 (1H, q-t, J_{20-21}=7.1 Hz, J_{20-16}=2.3 Hz, C_{20}–H), 4.70 (1H, sept, $J_1=J_{1'}$= 10.5 Hz, $J_2=J_{2'}$=5.3 Hz, 3β-H), 2.34—2.08 (2H, br s, C_{16}–H), 2.00 (3H, s, CH_3COO), 1.54 (3H, d-t, J_{21-20}= 7.0 Hz, J_{21-16}=1.6 Hz, 21-CH_3), 0.96 (3H, s, 19-CH_3), 0.71 (3H, s, 18-CH_3).

5β-Pregn-17(20)-en-3α-ol (13a)——One hundred mg of **13b** was dissolved in 10 ml of 1 N KOH in 95% ethanol and the solution was allowed to stand overnight at room temperature. It was then diluted with 100 ml of ether, washed with water, dried, and concentrated. The product (87 mg) was recrystallized from *n*-hexane to give compound **13a** as fine needles, mp 128—131°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48); C, 83.38; H, 11.33. Found: C, 83.46; H, 11.28. IR: 3320, 2975, 2920, 1450. NMR ($CDCl_3$) δ: 5.00 (1H, q-t, J_{20-21}= 7.0 Hz, J_{20-16}=2.3 Hz, C_{20}–H), 3.62 (1H, sept, $J_1=J_{1'}$=10.2 Hz, $J_2=J_{2'}$=5.6 Hz, 3β-H), 2.30—2.01 (2H, br s, C_{16}–H), 1.50 (3H, d-t, J_{21-20}=7.2 Hz, J_{21-16}=1.6 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.73 (3H, s, 18-CH_3). MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$].

Wittig Reaction of 3α-Hydroxy-5β-androstan-17-one (17)——Eight hundred mg of sodium hydride (50% dispersion in mineral oil) was washed 3 times with *n*-hexane and blown dry with nitrogen. Dry dimethyl sulfoxide (DMSO, 15.4 ml) was added and the mixture was heated with stirring under nitrogen at 75°C. After 45 min, the solution was cooled to room temperature and 8.1 g of ethyltriphenylphosphonium iodide in 30 ml of DMSO was added rapidly. The mixture was heated with stirring under nitrogen at 75°C. After 30 min, the solution was cooled to room temperature and a solution of **17** (1.06 g) in 30 ml of dry DMSO was added. The whole was again heated under nitrogen at 60°C. After 5 h, the mixture was cooled and poured onto ice water. The mixture was extracted with ether and after being washed with water, the extract gave a crystalline material (1.53 g), which was recrystallized from methanol as fine needles (587 mg), mp 185—187°C. *Anal.* Calcd for $C_{21}H_{34}O$ (302.48): C, 83.38; H, 11.33. Found: C, 83.18; H, 11.50. IR: 3275, 2925, 1740, 1600, 1465, 1450, 1365. NMR ($CDCl_3$) δ: 5.14 (1H, q-t, J_{20-21}=7.1 Hz, J_{20-16}=1.9 Hz, C_{20}–H), 3.65 (1H, sept, $J_1=J_{1'}$=10.5 Hz, $J_2=J_{2'}$=5.5 Hz, 3β-H), 2.36—2.16 (2H, m, C_{16}–H), 1.65 (3H, d-t, J_{21-20}= 7.0 Hz, J_{21-16}=2.0 Hz, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.86 (3H, s, 19-CH_3). Irradiation at 5.14 ppm changed the peaks at 1.64 ppm to a triplet, J=2.0 Hz. MS *m/e*: 302 (M^+), 287 (M^+-CH_3), 284 (M^+-H_2O), 269 [$M^+-(CH_3+H_2O)$]. The rt_R of this material is compared with that of the hydrolyzate components in Fr. 19 and Fr. 21 in Table II.

3α-Acetoxy-5β-pregn-17(20)-ene (14b)——The synthetic **14a** (67 mg) was treated with acetic anhydride in pyridine to give crude acetate (67 mg), which was recrystallized from methanol to give compound **14b** as fine needles, mp 115—119°C. *Anal.* Calcd for $C_{23}H_{36}O_2$ (344.52): C, 80.18; H, 10.53. Found: C, 80.04; H, 10.66. IR: 2940, 2860, 1735, 1450, 1380—1360, 1260—1240. NMR ($CDCl_3$) δ: 5.11 (1H, q-t, J_{20-21}= 7.1 Hz, J_{20-16}=2.0 Hz, C_{20}–H), 4.72 (1H, sept, $J_1=J_{1'}$=10.2 Hz, $J_2=J_{2'}$=5.1 Hz, 3β-H), 2.03 (3H, s, CH_3-COO), 1.65 (3H, d-t, J_{20-21}=7.1 Hz, J_{21-16}=2.0 Hz, 21-CH_3), 0.94 (3H, s, 19-CH_3), 0.86 (3H, s, 18-CH_3). The relative retention times of this synthetic steroid and acetylated Fr. 19 and Fr. 21 are compared in Table III.

Potassium 5β-pregn-3-en-20α-yl Sulfate (10c)——Chlorosulfonic acid (0.2 ml) was added to a solution of **10a** (33 mg) in pyridine (5 ml). The mixture was heated for 1 h at 60°C. Pyridine was removed under reduced pressure to give a residue, which was dissolved in 20 ml of water. After being made alkaline (pH, about 10) by adding 0.1 N KOH, the mixture was extracted with *n*-butanol. The combined extract was washed with water, and concentrated under reduced pressure at 50°C. The product (40 mg), obtained as a white powder, was recrystallized from methanol to give compound **10c** as needles, mp 134—135°C. *Anal.*

Calcd for $C_{21}H_{33}KO_4S \cdot H_2O$: C, 57.50; H, 8.04; S, 7.31. Found: C, 57.19; H, 7.96; S, 7.11. IR: 3500—3450, 2950, 2850, 1450, 1380, 1240—1230. NMR (CD_3OD) δ: 5.72—5.50 (1H, m, C_3–H), 5.40—5.20 (1H, m, C_4–H), 4.50—4.30 (1H, m, C_{20}–H), 1.37 (3H, d, J=5.6 Hz, 21-CH_3), 0.97 (3H, s, 19-CH_3), 0.74 (3H, s, 18-CH_3).

Potassium 5β-Pregn-2-en-20α-yl Sulfate (11c)——In the same manner as described for **10c**, the sulfate **11c** (18 mg) was obtained from 15 mg of **11a**. Recrystallization from methanol gave fine needles, mp 142—144°C. *Anal.* Calcd for $C_{21}H_{33}KO_4S \cdot H_2O$: C, 57.50; H, 8.04; S, 7.31. Found: C, 57.96; H, 8.29; S, 7.50. IR: 3450, 2940, 2870, 1445, 1380, 1240—1210. NMR (CD_3OD) δ: 5.55 (2H, br s, C_2– and C_3–H), 4.50—4.20 (1H, m, C_{20}–H), 1.37 (3H, d, J=6.2 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 0.73 (3H, s, 18-CH_3).

Hydrolysis of 10c and 11c——To refluxing aqueous solutions of **10c** and **11c** (each 2 mg in 5 ml) was added 5 ml of refluxing 6 N HCl, and the mixtures were refluxed for 30 min. After being cooled to room temperature, these reaction mixtures were extracted with ether. The combined extracts were washed with water, dried, and concentrated. Oily products, 1.2 mg from **10c** and 1.3 mg from **11c**, were obtained, which were subjected to gas chromatographic analyses.

Gas Chromatographic Comparison of Fr. 1 with Synthetic Steroidal Dienes——The relative retention times of steroidal dienes obtained by the hydrolysis of **2** (Fr. 1), **10c**, and **11c**, are shown below. Olefin **7a** was used as an internal standard, the retention time of which was 7.02 min (=1.00).

Hydrolyzate from	% of diene fraction	Relative retention times						
2	1.4	0.45	0.48	0.50	0.54	0.56	0.61	0.66
10c	32.4	0.46	0.49		0.54		0.60	
11c	36.3		0.48	0.50		0.57		0.68

All the peaks listed above showed parent ions at *m/e* 284 in GC–MS.

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